

10/536955

**REPLICATION OF HEPATITIS C VIRUS IN NON-HEPATIC  
EPITHELIAL AND MOUSE HEPATIC CELLS**

Qing Zhu

Ju-Tao Guo

Christoph Seeger

5

This application claims priority to U.S. provisional application 60/433,303, filed December 13, 2002, the entire contents of which are incorporated by reference  
10 herein.

**GOVERNMENT RIGHT**

Pursuant to 35 U.S.C. Section 202(c), it is acknowledged that the United States government has  
15 certain rights in the invention described herein, which was made in part with funds from the National Institutes of Health Grant No. AI48046.

**FIELD OF THE INVENTION**

20 This invention relates to the fields of molecular biology and pathology. Novel animal cell lines and non-hepatic human epithelial cell lines for the replication of hepatitis C virus (HCV), as well as methods for screening for anti-HCV drugs or HCV receptors using these  
25 cell lines are disclosed. Furthermore, adaptive sequence mutations in the HCV genome, which permit replication in non-human, and non-hepatic cell lines are also provided.

**BACKGROUND OF THE INVENTION**

30 Several publications and patent documents are cited in this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these citations is incorporated by reference herein.

35 Hepatitis C virus (HCV) is an enveloped, positive

stranded RNA virus that belongs to the *Flaviviridae*, a family of viruses including human pathogens such as yellow fever virus, dengue virus and West Nile virus (Q. L. Choo *et al.*, *Science* 244, 359-62 (1989)). Although  
5 broad tissue and species tropisms are hallmarks of these viruses, HCV replication has so far only been detected in human and chimpanzee livers. Moreover, for reasons that are not yet understood, HCV RNA levels in infected liver tissue are extremely low, generally below one copy of RNA  
10 per cell and hence, can only be detected with PCR, making it difficult to determine whether secondary sites for viral replication exist (J. Boisvert *et al.*, *J Infect Dis* 184, 827-35 (Oct 1, 2001); R. E. Lanford, *et al.*, *J Virol* 69, 8079-83 (1995)).

15 HCV encodes a polyprotein that is processed proteolytically into ten polypeptides (K. E. Reed, C. M. Rice, *Curr Top Microbiol Immunol* 242, 55-84 (2000)). Three of them are structural proteins required for capsid formation (core) and assembly into enveloped viral  
20 particles (E1 and E2). Four of them are enzymes including cysteine and serine proteases (NS2 and NS3), an ATP dependent helicase (NS3) and a RNA-directed RNA polymerase (NS5B). The functions of the remaining three polypeptides, p7, NS4B, and NS5A, for viral replication  
25 are not yet known. For study of replication of HCV in tissue culture cells, the structural proteins can be replaced with a selectable marker, such as the neomycin phosphotransferase. See for example Figure 2, left panel of Lohman *et al.* (V. Lohmann *et al.*, *Science* 285, 110-3  
30 (1999)). Replication of such subgenomic HCV replicons in tissue culture cells has so far only been demonstrated in the human hepatoma cell line Huh7, consistent with the narrow host and tissue tropism of HCV infections.

HCV infection poses a significant public health  
35 problem. Approximately 3% of the world's population has

persistent HCV infection. In 1989, the virus was identified as the major aetiological agent responsible for post-transfusion non-A and non-B hepatitis. Following primary HCV infection, persistent viraemia and chronic hepatitis develop in the majority of cases. Efforts to elucidate the mechanisms behind viral persistence and hepatocellular damage have been frustrated by the lack of a reliable cell culture system for viral propagation *in vitro*. In addition, as the chimpanzee is the only experimental animal susceptible to HCV infection, progress in research is hampered by the lack of a small animal model to facilitate pathophysiological studies as well as the evaluation of antiviral treatment and vaccine strategies.

Furthermore, although the initial HCV infection is asymptomatic, subsequent clinical manifestations of HCV induced liver disease include fibrosis, cirrhosis, and hepatocellular carcinoma (Alter, H. J., and L. B. Seeff. 2000. *Semin. Liver Dis.* 20:17-35). Combination antiviral therapy with alpha interferon (IFN- $\alpha$ ) and ribavirin, a purine nucleoside analogue, arrests disease progression and can lead to sustained recovery in only 45 to 80% of treated patients (Di Bisceglie, A. M., and J. H. Hoofnagle. 2002. *Hepatology* 36:S121-S127). Additionally, response to IFN- $\alpha$  therapy can vary significantly depending on the viral genotype, ranging from 30 to 40% for genotype 1 to as high as 80% for genotypes 2 and 3. This suggests that viral determinants also play an important role in regulating the cellular IFN response against HCV (Kinzie, J. L., et al., 2001. *J. Viral Hepatitis* 8:264-269; McHutchison, J. G., et al., 1998. *N. Engl. J. Med.* 339:1485-1492). The parameters determining the success or failure of antiviral therapy are not understood, and their identification represents a major challenge in HCV biology.

Therefore, there is a desperate need for non-hepatic cell culture systems, and small animal models for the identification and characterization of anti-viral agents for the prevention and treatment of HCV infection.

5 Additionally, there is a need in the art to elucidate the mechanism of HCV inhibition by IFN- $\alpha$ , so that other treatments may be found.

#### **SUMMARY OF THE INVENTION**

10 The present invention provides HCV replicating cells and cell lines derived from human non-hepatic cells or non-human cells. According to one embodiment of the invention, the cells are human epithelial cells of non-liver origin, such as, HeLa cells. According to another  
15 embodiment of the invention, the cells capable of replicating HCV are hepatoma and hepatocyte cells of mouse origin, such as, Hepa1-6 cells, or AML12 cells respectively.

The present invention also provides a non-human host  
20 animal comprising cells infected with HCV. In one embodiment of the invention, the host animal is a mouse. In another embodiment of the invention, the cells infected with HCV are mouse hepatoma cells.

Also provided by the present invention are methods  
25 for producing human non-hepatic cells or non-human cells that are capable of replicating HCV, and cell lines comprising the same. Such methods include transfection with total HCV RNA or an HCV replicon which comprises one or more adaptive mutations which facilitate replication  
30 in a cell of interest.

The present invention further provides methods for screening an agent that modulates HCV replication by incubating the agent with the aforementioned cells or administering the agent to the aforementioned host animal  
35 comprising cells replicating HCV and assessing said agent

for modulation of HCV replication. Such agents may inhibit or enhance production of HCV. These agents may be cytopathic or non-cytopathic to HCV infected cells. Agents which activate aspects of the JAK/STAT pathway may  
5 also be screened using the cells and cell lines of the invention.

Also provided by the present invention are HCV derived polynucleotides comprising adaptive mutations. The present inventor has discovered that these mutations  
10 are associated with expanded tropism of HCV.

Additionally, the present invention provides polypeptides encoded by the mutated HCV polynucleotides described above.

#### 15 BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are a Northern blot (Fig. 1A) and micrographs showing replication of HCV subgenomic replicons in HeLa cells. (Fig. 1A) Detection of HCV viral RNA. Total RNA (5 µg) was isolated from HeLa cell  
20 lines (SL1, SL3-7) that were established from G418 resistant cell colonies and analyzed by Northern blot analysis. Blots were hybridized with radiolabeled RNA probes corresponding to the HCV NS5 region to detect viral RNA (vRNA) and the ΔE1 region of human papilloma  
25 virus (HPV) present in HeLa cells. *In vitro* transcribed HCV RNA (1 ng, + 5µg total RNA from Huh7 cells, lane 7) served as a marker (M) and control for the hybridization reaction and 28S ribosomal RNA as a control for the amount of RNA present in each sample analyzed. GS4.1 is a  
30 Huh7 derived cell line expressing HCV subgenomic replicons. RNA in SL1 cells was analyzed from cells harvested at the indicated passage (p). SL3-7 were analyzed at p3 (Fig. 1B). Immunohistochemical analysis of HCV replication in HeLa cells. Expression of NS5A in  
35 GS4.1 and SL1 cells (p26) was detected with a monoclonal

antibody bound to fluorescent isothiocyanate (FITC)-conjugated antibody. Parental Huh7 and HeLa cells served as controls.

Figure 2 diagrams sequence analysis of HCV subgenomes in HeLa and mouse hepatoma Hepal-6 cells. (Left section) The physical map of HCV subgenomic RNA include the positions of the first amino acid NS3 and the last residue of the polyprotein. The internal ribosomal entry site for the translation of the NS genes is indicated (EMCV-IRES). (Right section) Mutations causing amino acid changes identified in cDNAs isolated from subgenomic replicons present in GS4.1 and the indicated HeLa (SL1 (p26); SL2, p5) and Hepal-6 cell lines (MH1 (p12), MH2 (p4), MH4 (p4)) are depicted with horizontal bars. Four independent clones were sequenced from each PCR fragment that was amplified from cDNAs obtained from total RNA purified from the indicated cell lines. Mutations present in more than one cell line are indicated by amino acid position. Mutations that occurred in 50% of the clones analyzed are marked with an asterisk. Mutations that occurred in only one of four clones analyzed were not included in the figure. A deletion identified in cDNA clones obtained from SL1 cells spanning amino acids 2371 to 2413 is indicated ( $\Delta$ ).

Figures 3A and 3B are a Northern blot and micrographs showing replication of HCV subgenomes in mouse hepatoma cells. (A) Detection of HCV RNA. RNA in Hepal-6 cell lines (MH1-5) that were established from G418 resistant cell colonies and analyzed as described in the legend to Figure 1 except that a radiolabeled probe specific to mouse albumin cDNA was used in lieu of the probe against HPV. MH4-5 were analyzed at p4 (B). Immunohistochemical analysis of HCV replication in MH1 cells (p3). Expression of NS5A was detected as described in Figure 1. Hepal-6 cells served as a

negative control.

Figure 4 is a Northern blot showing detection of HCV RNA in mouse hepatocyte cells. AML12 cells were transfected with total RNA from GS4.1 cells, which  
5 express subgenomic HCV replicons, and HFL cells, which express full length HCV genomes. 5 micrograms of total RNA was isolated from AML12 cell lines (MA6-5 to MA6-8, and MAC1) that were established from G418 resistant cell colonies and analyzed by Northern blot analysis. sgrNA  
10 indicates subgenomic RNA and flrNA full length genomic RNA.

Figures 5A and 5B are two Northern blots showing the antiviral activity of the HCV RNA polymerase inhibitor 2'-C-methyladenosine (2CMA). GS4.1 (Huh7) cells (5A) and  
15 SL1 (HeLa) cells (5B) were treated with 10  $\mu$ M 2CMA. The cells were harvested at the indicated time points. Total cellular RNA was extracted and viral RNA (vRNA) analyzed by Northern blot analysis.

Figures 6A and 6B are two graphs depicting antiviral  
20 activity of the HCV RNA polymerase inhibitor 5-OH-cytidine. GS4.1 (Huh7) cells and SL1 (HeLa) cells were treated with the indicated amounts of 5-OH-cytidine. The DNA polymerase inhibitor 5-OH-deoxy-cytidine was used as a negative control. The cells were harvested 72 hours  
25 after incubation with the drugs. Total cellular RNA was extracted and viral RNA analyzed by Northern blot analysis. The intensity of the bands corresponding to HCV RNA was determined with a Fuji phosphoimager.

Figures 7A and 7B are a Northern blot and a graph  
30 showing antiviral activity of IFN- $\alpha$  in Huh 7 (GS4.1) and HeLa (SL1) cell lines containing HCV replicons. (7A) Viral RNA (vRNA) levels present in GS4.1 and SL1 cells incubated with 0, 0.1, 0.3, 1, 3, 10, 30, and 100 IU of IFN-  $\alpha$  /ml (lanes 1 to 9 and 12 to 18) and with 0.01 and  
35 0.03 IU/ml (lanes 10 and 11) for 72 h were determined by

Northern blot analysis. rRNA (28S rRNA) served as a control for the amount of RNA loaded per lane. (7B) Amounts of HCV RNA were determined with a Fuji phosphorimager, and the values were plotted as the percentages of the values obtained with untreated cells in lanes 1 and 9.

Figures 8A and 8B are micrographs and histograms showing that IFN-  $\alpha$  induces apoptosis of SL1 cells. (8A) Annexin V-FITC staining. SL1 cells grown on glass coverslips were left untreated (upper left) or treated with 100 IU of IFN-  $\alpha$  /ml for 6 h (upper right) or 20 h (lower left) or with 100 IU of IFN-  $\alpha$  /ml and 20  $\mu$ M caspase inhibitor ZVAD-FMK for 20 h (lower right). Cells were then processed for annexin V FITC staining and viewed with a fluorescence microscope. (8B) Flow cytometry analysis. SL1 cells were left untreated (upper left) or treated with 100 IU of IFN-  $\alpha$  /ml (upper right) or with IFN-  $\alpha$  and 20  $\mu$ M ZVAD-FMK (lower left) for 24 h. To inhibit viral replication, SL1 cells were incubated at 39°C for 60 h and then treated with 100 IU of IFN-  $\alpha$  /ml for 24 h (lower left). Cells were harvested and processed for annexin V-FITC staining and analyzed by flow cytometry. The percentages of FITC-positive cells are indicated.

Figures 9A and 9B are Northern blots and a graph showing a comparison of IFN-  $\alpha$  responses against HCV and flavivirus Kunjin virus replicons in HeLa cells. (9A) SL1 and KUNCD20 cells were incubated with 0, 0.01, 0.04, 0.16, 0.625, 2.5, 10, 40, and 160 IU of IFN-  $\alpha$  /ml (lanes 1 to 9 and 10 to 18, respectively) for 72 h, and viral RNA levels were determined by Northern blot analysis with a plus-strand-specific RNA probe for the neomycin phosphotransferase II gene. Mx-1 mRNA served as a control for IFN-  $\alpha$  -induced gene expression.  $\beta$ -Actin mRNA served as a control for the amount of RNA loaded per lane. (9B)



The amounts of HCV and Kunjin virus replicon RNA (arbitrary units) were determined with a Fuji phosphorimager.

Figure 10 shows an overview of the interactions between a virus and the IFN system. Replication of viruses in cells produces dsRNA and viral proteins, which activate PKR and OAS/RNase L antiviral pathways and also signal to the promoter of the IFN- $\beta$  gene by activating transcription factors IRF3, NF- $\kappa$ B, and ATF2. Secreted IFN binds to its receptor and activates receptor-associated Jak kinases, leading to the formation of the trimeric transcription factor ISGF3, which binds to the IFN-stimulated response element (ISRE) on promoters of IFN-stimulated genes. Among the products of the several hundred genes induced by IFN, PKR, OAS/RNase L, and Mx are the best-characterized antiviral proteins, which inhibit different stages of viral replication and induce apoptosis of virally infected cells.

Figures 11A-11D are Northern blots and two graphs showing inhibition of the IFN- $\alpha$  response by genistein and the V protein of HPIV2. (11A) GS4.1 cells were incubated with 100  $\mu$ g of genistein/ml for 2 h and then with 100 IU/ml IFN- $\alpha$  for an additional 24 h. Viral RNA levels were determined by Northern blot analysis. The cells were harvested at the indicated time points, and Mx-A mRNA and viral RNA levels were determined by Northern blot analysis. Ribosomal 28S RNA was used as a control for the amount of RNA loaded on each lane. (11B) The amounts of viral RNA were measured with a phosphorimager and plotted as percentages of the values obtained with untreated cells. (11C) GS4.1 cells were transfected with pCMV-E3L and pEF-HA-HPIV2 and treated with IFN- $\alpha$  at the indicated concentrations for 3 days. HCV RNA was subjected to Northern blot analysis. (11D) Viral RNA levels were determined with a Fuji

phosphorimager and plotted as the percentages of the values obtained with untreated cells.

Figures 12A and 12B show the dsRNA response in parental Huh7 and HeLa cells and HCV replicon-containing GS4.1 and SL1 cells. (12A) Phosphorylation of eIF-2 $\alpha$ . Huh7, GS4.1, HeLa, and SL1 cells were left untreated (lanes 1, 5, 9, and 13) or treated with 100 IU of IFN- $\alpha$  /ml for 12 h (lanes 2, 4, 6, 8, 10, 12, 14, and 16) and then transfected with poly(I:C) and incubated for 3 h (lanes 3, 4, 7, 8, 11, 12, 15, and 16). eIF-2 $\alpha$  -P and total eIF-2  $\alpha$  were determined by Western blots analysis with a monoclonal antibody specific for eIF-2 $\alpha$  -P and an antibody specific for total eIF-2 $\alpha$  protein. (12B) Induction of IFN- $\beta$  mRNA by dsRNA and IFN- $\alpha$ . Parental Huh7 and HeLa cells and HCV replicon-containing GS4.1 and SL1 cells were left untreated (lanes 1, 5, 9, and 13) or treated with 100 IU of IFN- $\alpha$  /ml (lanes 3, 4, 7, 8, 11, 12, 15, and 16) for 12 h and then transfected with poly(I:C) (lanes 2, 4, 6, 8, 10, 12, 14, and 16) for 3 h. An RNase protection assay was performed with probes specific for IFN- $\beta$  and  $\beta$ -actin mRNAs.

Figures 13A-13C show dose-dependent inhibition of the IFN- $\alpha$  response against subgenomes by lactacystin and epoxomicin. (13A) Cells were incubated with lactacystin and epoxomicin at the indicated concentrations for 7 h and then for an additional 12 h without the drugs. One hour after incubation with the proteasome inhibitors, IFN- $\alpha$  (100 IU/ml) was added for 6 h to a fraction of the cell culture plates (lanes 6 to 10 and 16 to 20). Viral RNA levels were determined by Northern blot analysis. rRNA was used as a control for the amount of RNA present in the samples. (13B and 13C) The amount of viral RNA was measured with a phosphorimager, and values were plotted as percentages of the values obtained with untreated cells.

Figures 14A and 14B are Northern blots and a graph showing that proteasome inhibitors block the IFN- $\alpha$  response against HCV replicons. (14A) GS4.1 cells were left untreated (lanes 1 to 3 and 19 to 21) or treated with 100 IU of IFN- $\alpha$  /ml for 6 h (lanes 4 to 6 and 22 to 24), with 5  $\mu$ M lactacystin (lanes 7 to 9 and 25 to 27) or 1  $\mu$ M epoxomicin (lanes 13 to 15 and 31 to 33) alone for 7 h, or with 5  $\mu$ M lactacystin (lanes 10 to 12 and 28 to 30) or 1  $\mu$ M epoxomicin (lanes 16 to 18 and 34 to 36) alone for 1 h and then in the presence of 100 IU of IFN- $\alpha$  /ml for an additional 6 h. Cells were harvested at 12 h (lanes 1 to 18) and 18 h (lanes 19 to 36) after addition of the cytokine. Viral RNA levels were determined by Northern blot analysis. rRNA was used as a control for the amount of RNA present in the samples. (14B) The amount of viral RNA was measured with a phosphorimager and the mean values and standard deviations from three samples were plotted. \*, P < 0.05; \*\*, P < 0.01. PSL, arbitrary units.

Figures 15A and 15B show a Northern blot graph demonstrating that proteasome inhibitors prevent establishment of an IFN- $\alpha$  response against HCV replicons. (15A) GS4.1 cells were treated with IFN- $\alpha$  for 10 h, followed by treatment with the indicated proteasome inhibitors for 12 h. Cells were left untreated (lanes 1 to 3 and 4 to 6) or treated with 100 IU of IFN- $\alpha$  /ml for 10 h (lanes 7 to 18), followed by treatment with 10  $\mu$ M lactacystin (lanes 13 to 15) or 1  $\mu$ M epoxomicin (lanes 16 to 18) for 12 h. Cells were harvested at 0, 10, and 18 h after the cytokine treatment, as indicated. Viral RNA levels were determined by Northern blot analysis. rRNA was used as a control for the amount of RNA present in the samples. (15B) The amount of viral RNA was measured with a phosphorimager and the mean values and standard deviations from three samples were plotted. PSL,

arbitrary units.

#### DETAILED DESCRIPTION OF THE INVENTION

The hepatitis C virus (HCV) pandemic affects the  
5 health of more than 170 million people and is the major  
indication for orthotopic liver transplantations (OLT).  
Although the human liver is the primary site for HCV  
replication, it is not known whether extrahepatic tissues  
are also infected by the virus and whether non-primate  
10 cells are permissive for RNA replication. However,  
because viral replication leads to the accumulation of  
mutations, it is conceivable that variants can emerge  
with novel properties such as the potential to replicate  
in different cell types of various species. Furthermore,  
15 accumulation of a large number of quasispecies may also  
contribute to resistance to IFN- $\alpha$  treatment. Therefore,  
it is important to determine the properties of HCV  
variants, and the effect such variation has on the  
efficacy of IFN- $\alpha$  therapy.

20 Provided herein is evidence that subgenomic HCV RNAs  
can replicate in mouse hepatoma and non-hepatic human  
epithelial cells. Moreover, efficient replication  
requires adaptation of the virus to cell-type specific  
environmental conditions. These results show that HCV  
25 RNA replication can lead to the accumulation of mutants  
with altered tissue and host tropism thereby facilitating  
the development of small animal models for HCV infection.

In accordance with the present invention, there are  
provided nucleic acids and stably-transfected human non-  
30 hepatic, and murine hepatic cell lines that replicate  
HCV. Also provided are methods of use for such cells for  
identifying therapeutic anti-viral agents for the  
treatment of HCV infection. Additionally, the  
availability of a murine line which replicates HCV  
35 enables the production of a greatly needed mouse model of

HCV infection. Furthermore, the invention provides polynucleotides and their corresponding polypeptides which have adaptive mutations which result in expanded tropism of HCV.

5       The detailed description set forth below describes preferred methods for making and using the nucleic acids and cell lines of the present invention, and for practicing the methods of the invention. Any molecular cloning or recombinant DNA techniques not specifically  
10       described are carried out by standard methods, as generally set forth, for example, in Sambrook et al., "DNA Cloning, A Laboratory Manual," Cold Spring Harbor Laboratory, 1989 and Ausubel et al. Current Protocols in Molecular Biology, J. Wiley & Sons, 1995.

15

#### **I. Definitions**

The following definitions are provided to aid in understanding the subject matter regarded as the invention.

20       As used herein, "hepatitis C virus" or "HCV" shall mean any representative of a diverse group of related viruses classified within the hepacivirus genus of the Flaviviridae family.

25       "Anti-HCV compounds" may include any inhibitor of HCV-derived enzymes, such as protease, helicase, and polymerase inhibitors. Anti-HCV compounds also include IRES inhibitors, glycosylation inhibitors, and molecules which block the HCV receptor (thus preventing entry into cells.) Other anti-HCV compounds include compounds which  
30       enhance the specific or non-specific immune response, thereby ameliorating HCV infection or symptoms.

      "HCV replication levels" may be measured by methods known in the art, including but not limited to detection of replicated HCV replicons, HCV NS protein production,  
35       or incorporation of detectably labeled nucleotides into

an HCV nucleic acid.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism or virus in which it originated. When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues), and explicitly includes viral RNA. An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

"RNA subgenome" refers to any molecule which lacks some portion of a genome. For example, an RNA subgenome can be an HCV RNA molecule in which a structural gene has been replaced with a selection agent.

All amino acid residue sequences represented herein conform to the conventional left-to-right amino-terminus to carboxy-terminus orientation.

The term "isolated protein" or "isolated and purified protein" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention.

Alternatively, this term may refer to a protein that has been sufficiently separated from other proteins with which it would naturally be associated, so as to exist in "substantially pure" form. "Isolated" is not meant to  
5 exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, for example, due to incomplete purification, addition of stabilizers, or compounding  
10 into, for example, immunogenic preparations or pharmaceutically acceptable preparations.

"Variants", "mutants" and "derivatives" of particular sequences of nucleic acids refer to nucleic acid sequences that are closely related to a particular  
15 sequence but which may possess, either naturally or by design, changes in sequence or structure. By closely related, it is meant that at least about 75%, but often, more than 90%, of the nucleotides of the sequence match over the defined length of the nucleic acid sequence.  
20 Changes or differences in nucleotide sequence between closely related nucleic acid sequences may represent nucleotide changes in the sequence that arise during the course of normal replication or duplication in nature of the particular nucleic acid sequence. Other changes may  
25 be specifically designed and introduced into the sequence for specific purposes, such as to expand the tropism of viral RNA, or to change an amino acid codon or sequence in a regulatory region of the nucleic acid. Such specific changes may be made in vitro using a variety of  
30 mutagenesis techniques or produced in a host organism placed under particular selection conditions that induce or select for the changes. Such sequence variants generated specifically may be referred to as "mutants" or "derivatives" of the original sequence. The terms  
35 "percent similarity", "percent identity" and "percent

homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

5 An "adaptive mutation" is a mutation in a nucleic acid sequence which produces a change in viral properties or activity. For example, an adaptive mutation includes, but is not limited to, a mutation which provides enhanced tropism for HCV, or which alters the efficacy of IFN- $\alpha$  treatment.

10 An HCV peptide, polypeptide, or protein of the invention includes any analogue, fragment, derivative or mutant which is derived from a HCV peptide or polypeptide and which retains at least one property or other characteristic of the HCV polypeptide. Different  
15 "variants" of the HCV polypeptide exist in nature. These variants may be alleles characterized by differences in the nucleotide sequences of the gene coding for the protein, or may involve different RNA processing or post-translational modifications. The skilled person can  
20 produce variants having single or multiple amino acid substitutions, deletions, additions or replacements. These variants may include inter alia: (a) variants in which one or more amino acids residues are substituted with conservative or non-conservative amino acids, (b)  
25 variants in which one or more amino acids are added to the HCV peptide or polypeptide, (c) variants in which one or more amino acids include a substituent group, and (d) variants in which one or more amino acids are deleted from the HCV peptide or polypeptide. Other HCV peptides  
30 or polypeptides of the invention include variants in which amino acid residues from one species are substituted for the corresponding residue in another species, either at the conserved or non-conserved positions. In another embodiment, amino acid residues at  
35 non-conserved positions are substituted with conservative



or non-conservative residues. The techniques for obtaining these variants, including genetic ( suppressions, deletions, mutations, etc. ), chemical, and enzymatic techniques are known to the person having  
5 ordinary skill in the art.

To the extent such variations, analogues, fragments, derivatives, mutants, and modifications, including alternative nucleic acid processing forms and alternative post-translational modification forms result in  
10 derivatives of the HCV peptide or polypeptide that retain any of the biological properties of the HCV peptide or polypeptide, they are included within the scope of this invention.

The term "functional" as used herein implies that  
15 the nucleic or amino acid sequence is functional for the recited assay or purpose.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given sequence.  
20 For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the fundamental and novel characteristics of the sequence.

A "replicon" is any genetic element, for example, a  
25 plasmid, cosmid, bacmid, phage or virus, that is capable of replication largely under its own control. A replicon may be either RNA or DNA and may be single or double stranded.

A "vector", is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence  
30 or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

The phrase "operably linked" when referring to  
35 nucleic acid constructs is used herein to indicate that

the respective promoter, operator and coding sequences, as well as any other 5' and 3' regulatory sequences, are arranged in the appropriate location, order and reading frame such that the desired control (e.g., expression) is  
5 effected under appropriate conditions.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than  
10 three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either  
15 RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either  
20 single-stranded or double-stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide  
25 probe typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be  
30 sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a  
35 non-complementary nucleotide fragment may be attached to

the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided  
5 that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "specifically hybridize" refers to the association between two single-stranded nucleic acid  
10 molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a  
15 substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence. For example, hybridizations  
20 may be performed, according to the method of Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989), using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium  
25 pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3)  
30 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between  
35 nucleic acid molecules of a specified sequence homology

(Sambrook et al., 1989) is as follows:

$$T_m = 81.5^{\circ}\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\#\text{bp in duplex}$$

5

As an illustration of the above formula, using  $[\text{Na}^+] = [0.368]$  and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the  $T_m$  is  $57^{\circ}\text{C}$ . The  $T_m$  of a DNA duplex decreases by 1 -  $1.5^{\circ}\text{C}$  with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridization temperature of  $42^{\circ}\text{C}$ .

The stringency of the hybridization and wash depend primarily on the salt concentration and temperature of the solutions. In general, to maximize the rate of annealing of the probe with its target, the hybridization is usually carried out at salt and temperature conditions that are  $20\text{-}25^{\circ}\text{C}$  below the calculated  $T_m$  of the hybrid. Wash conditions should be as stringent as possible for the degree of identity of the probe for the target. In general, wash conditions are selected to be approximately  $12\text{-}20^{\circ}\text{C}$  below the  $T_m$  of the hybrid. In regards to the nucleic acids of the current invention, a moderate stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and  $100 \mu\text{g/ml}$  denatured salmon sperm DNA at  $42^{\circ}\text{C}$ , and washed in 2X SSC and 0.5% SDS at  $55^{\circ}\text{C}$  for 15 minutes. A high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and  $100 \mu\text{g/ml}$  denatured salmon sperm DNA at  $42^{\circ}\text{C}$ , and washed in 1X SSC and 0.5% SDS at  $65^{\circ}\text{C}$  for 15 minutes. A very high stringency hybridization is defined as hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and  $100 \mu\text{g/ml}$  denatured salmon sperm DNA at  $42^{\circ}\text{C}$ , and washed in 0.1X SSC and 0.5% SDS at  $65^{\circ}\text{C}$  for 15 minutes.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when  
5 expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radioimmunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or  
10 double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter  
15 gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like.

The terms "transform", "transfect", "transduce",  
20 shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, electroporation, microinjection, PEG-fusion and the like.

25 The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or  
30 independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on or inherited to progeny cells or organisms of  
35 the recipient cell or organism. In other applications,

the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth in vitro for many generations.

A "selectable marker" or a "selection agent" refers to a nucleic acid sequence that when expressed confers a selectable phenotype, such as antibiotic resistance, to a transformed cell.

A "viral antigen" shall be any peptide, polypeptide or protein sequence, segment or epitope that is derived from a virus that has the potential to cause a functioning immune system of a host to react to said viral antigen.

An "antibody" or "antibody molecule" is any immunoglobulin, including antibodies and fragments thereof, that binds to a specific antigen. The term includes polyclonal, monoclonal, chimeric, and bispecific antibodies. As used herein, antibody or antibody molecule contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule such as those portions known in the art as Fab, Fab', F(ab')<sub>2</sub> and F(v).

The term "detectable label" is used herein to refer to any substance whose detection or measurement, either directly or indirectly, by physical or chemical means, is indicative of the presence of the target bioentity in the test sample. Representative examples of useful detectable labels, include, but are not limited to the following: molecules or ions directly or indirectly detectable based on light absorbance, fluorescence, reflectance, light scatter, phosphorescence, or

luminescence properties; molecules or ions detectable by their radioactive properties; molecules or ions detectable by their nuclear magnetic resonance or paramagnetic properties. Included among the group of molecules indirectly detectable based on light absorbance or fluorescence, for example, are various enzymes which cause appropriate substrates to convert, e.g., from non-light absorbing to light absorbing molecules, or from non-fluorescent to fluorescent molecules.

As used herein, the term "living host" shall mean any non-human autonomous being.

## **II. Methods for Obtaining HCV RNA and Producing Non-Hepatic Human Cell Lines and Non-Human Hepatic Cell Lines that Replicate HCV.**

The HCV replicating non-hepatic human cell-based and non-human hepatic cell-based systems are prepared according to the general methods set forth below for isolation of nucleic acids, transformation of cultured cells, and maintenance of cell lines.

### **A. Nucleic acids**

The HCV replicons of the present invention comprise adaptive mutations which alter the ability of HCV to replicate in different cell types. Surprisingly, the present inventors have identified mutations which are associated with expanded viral tropism.

The HCV nucleic acid molecules of the invention may be prepared by two general methods: (1) They may be synthesized from appropriate chemical starting materials, or (2) they may be isolated from biological sources. Both methods utilize protocols well known in the art.

The availability of nucleotide sequence information enables preparation of an isolated nucleic acid molecule of the invention by oligonucleotide synthesis. Synthetic



oligonucleotides may be prepared by the phosphoramidite method employed in the Applied Biosystems 38A DNA Synthesizer or similar devices. The resultant construct may be purified according to methods known in the art, such as high performance liquid chromatography (HPLC). Long, double-stranded polynucleotides, such as a DNA molecule of the present invention, must be synthesized in stages due to the size limitations inherent in current oligonucleotide synthetic methods. Thus, for example, a 3 kilobase double-stranded molecule may be synthesized as several smaller segments of appropriate complementarity. Complementary segments thus produced may be ligated such that each segment possesses appropriate cohesive termini for attachment of an adjacent segment. Adjacent segments may be ligated by annealing cohesive termini in the presence of DNA ligase to construct an entire 3 kilobase double-stranded molecule. A synthetic DNA molecule so constructed may then be cloned and amplified in an appropriate vector.

HCV nucleic acid sequences may be isolated from appropriate biological sources using methods known in the art. For example, total RNA can be extracted with TRIzol reagent from Gibco BRL, although other reagents are also available for this purpose.

In some cases, it may be desirable to synthesize HCV subgenomic RNA wherein a selectable marker gene is substituted for a HCV structural gene.

The availability of HCV replicon encoding nucleic acids enables the production of strains of laboratory mice carrying part or all of the HCV sequence or mutated sequences thereof. Such mice provide an *in vivo* model for studying HCV infection, and analyzing possible treatment modalities for the same.

Methods of introducing transgenes in laboratory mice are known to those of skill in the art. Three common

methods include: 1. integration of retroviral vectors encoding the foreign gene of interest into an early embryo; 2. injection of DNA into the pronucleus of a newly fertilized egg; and 3. the incorporation of  
5 genetically manipulated embryonic stem cells into an early embryo.

A transgenic mouse carrying an HCV replicon comprising the adaptive mutations is generated by genomic integration of exogenous genomic sequence encoding HCV.  
10 These transgenic animals are useful for drug screening studies as animal models for human diseases.

The term "animal" is used herein to include all vertebrate animals, except humans. It also includes an individual animal in all stages of development, including  
15 embryonic and fetal stages. A "transgenic animal" is any animal containing one or more cells bearing genetic information altered or received, directly or indirectly, by deliberate genetic manipulation at the subcellular level, such as by targeted recombination or  
20 microinjection or infection with recombinant virus. The term "transgenic animal" is not meant to encompass classical cross-breeding or *in vitro* fertilization, but rather is meant to encompass animals in which one or more cells are altered by or receive a recombinant DNA  
25 molecule. This molecule may be specifically targeted to a defined genetic locus, be randomly integrated within a chromosome, or it may be extrachromosomally replicating DNA. The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or  
30 genetic information was introduced into a germ line cell, thereby conferring the ability to transfer the genetic information to offspring. If such offspring, in fact, possess some or all of that alteration or genetic information, then they, too, are transgenic animals.

35 A type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells may be obtained from pre-implantation embryos cultured *in vitro* (Evans et

al., (1981) Nature **292**:154-156; Bradley et al., (1984) Nature **309**:255-258; Gossler et al., (1986) Proc. Natl. Acad. Sci. **83**:9065-9069). Transgenes can be efficiently introduced into the ES cells by standard techniques such as DNA transfection or by retrovirus-mediated transduction. The resultant transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The introduced ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal.

#### B. Cell lines

The cell lines of the invention include any cell which supports production of HCV components. These cells include human, non hepatic cells and/or non-human hepatic cells, such as murine hepatic cells. Cell lines useful for practice of the invention include, but are not limited to HELA, a non-hepatic epithelial cell line (ATCC CRL number CCL-2.2), Hepal-6, a murine hepatoma cell line (ATCC CRL number-1830), and AML-12, a murine hepatocyte cell line (ATCC CRL number-2254).

To achieve stable gene transfer, HCV subgenomic RNA is introduced into host cells. This may be accomplished according to numerous methods known in the art, including, but not limited to: (1) calcium phosphate transfection; (2) transfection with DEAE-dextran; (3) electroporation; and (4) liposome-mediated transfection. For general protocols, see, e.g., chapter 9 in Current Protocols in Molecular Biology, Ausubel et al. (editors), John Wiley & Sons, Inc. 1987-1995. For stable transfer of nucleic acids into mammalian cells, the liposome-mediated transfection method may be used in the present invention because of the large amount of nucleic acid that can be introduced into the cells, thereby increasing the possibility of integration of the nucleic acid into the host genome.

Cells are grown according to standard methods known in the art, such as those set forth in Culture of Animal Cells: A Manual of Basic Technique by R. Ian Freshney, 4th ed. Edition, available from the ATCC.

- 5       Stable transfectants are selected by the ability of an individual cell colony to grow in the presence of a selection agent, e.g., an antibiotic, by virtue of a resistance-encoding gene on HCV RNA or by isolating cells using FACS and antibodies directed any HCV protein.
- 10       Detection and quantitation of expression of HCV gene products in stably-transfected cell lines of the invention can be accomplished using a variety of known assays. For instance, cells transformed with the RNA subgenomes of HCV can be selected with an antibiotic such
- 15   as G418 (neomycin). Alternatively, cells may be selected based on the presence and accumulation of HCV RNA or HCV gene products. As another example, the starting HCV encoding nucleic acids may be modified to also comprise a hygromycin or puromycin or any other resistance or
- 20   reporter gene, such that cells transfected with the nucleic acids can be selected by their ability to grow on hygromycin- or puromycin-containing medium. Alternatively, selectable markers including luciferase, beta lactamase etc., may be utilized which allow for the
- 25   selection of cells by FACS and related procedures. In an alternative embodiment, a separate plasmid may be constructed that comprises an antibiotic resistance gene, and can be used to co-transfect cells along with the subgenomic RNA molecules. Further, as described in
- 30   detail in the following Examples, cells stably transfected with the subgenomic HCV RNA are grown in the appropriate medium for a selected period of time, the medium is then collected and analyzed for the presence of HCV RNA by dot blot hybridization or by conventional
- 35   Northern hybridization, using a radioactively labeled

probe having HCV DNA or RNA complementary sequences.  
Alternatively, viral gene products may be detected in the  
cells of the invention using conventional methods,  
including, without limitation, immunoassay and Western  
5 blotting.

Using the assays described above, stably-transfected  
cell lines can be selected which possess optimum  
characteristics for use in cell-based assays for  
screening potential anti-viral compounds.

10 Another aspect of the invention includes a non-human  
host animal which comprises the HCV expressing cells of  
the invention. These animals may be produced by  
administration of a HCV replicating cell, an HCV encoding  
nucleic acid having one or more adaptive mutations which  
15 permit replication in mice. The cells or viral nucleic  
acid could be directly injected intravenously (e.g. via  
tail vein injection), intramuscularly, subcutaneously, or  
via-intrahepatic injection. Alternatively, transgenic  
mice could be produced using the HCV replicons of the  
20 inventions, as described above.

### **III. Uses of Cell Lines for Cell-Based Assays of Potential Anti-HCV Agents**

The human non-hepatic and murine hepatic cell lines  
25 of the invention which replicate HCV may be used in  
research, diagnostic, and therapeutic applications,  
including cell-based assays to evaluate the effectiveness  
of potential anti-HCV compounds, utilizing methodologies  
known in the art. Typical assays are summarized herein  
30 below. These cell-based assays may be performed in  
standard cell culture media utilizing commonly-available  
equipment, reagents and culture containers.

Persons skilled in the art will appreciate that  
these assays represent exemplary embodiments, and may be  
35 varied to provide similar/equivalent equipment or

reaction conditions. For example, a variety of genes encoding antibiotic resistance are available, and can be utilized in accordance with the present invention in the generation of the cell lines of the invention. In a preferred embodiment, RNA isolated from parental human hepatic or untransformed cells is also utilized as a control in the assays described herein below to determine the effects of potential anti-viral compounds on HCV expressed in the cells. The control RNA is obtained in a manner similar to the HCV RNA. This cell line is treated in the assays described herein below as a negative control, to assure that any effects observed are due to the action of the compound being tested on HCV, and not non-specific effects due to the introduction of RNA into the cells.

#### **A. General Cell-Based Assay for Inhibitors of HCV replication**

96-well microtiter plates are seeded with an appropriate amount of cells which replicate HCV in a standard cell culture medium containing G418 (e.g., 400 µg/ml), as well as standard concentrations of penicillin, streptomycin and kanamycin or gentamicin to prevent bacterial and mycoplasma contamination. The cells are incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator. On day 0 wells are washed three times with warm phosphate-buffered saline (PBS). The culture medium is then replaced with fresh medium containing 0.3% dimethylsulfoxide (DMSO), 10% fetal calf serum (FCS), penicillin, streptomycin, kanamycin/gentamicin, containing one of the following ingredients: (1) various concentrations of a known HCV inhibitor, such as interferon alpha, as a positive control; and (2) various concentrations of one or more of the compounds to be tested. The plates are incubated at 37°C in humidified,

5% CO<sub>2</sub> incubator for 24, 48, and 72 hours. The plates are washed twice with PBS and then with a solution of methanol and acetone (1:1) to fix the cells. The cells are then incubated with an antibody specific for a viral protein (i.e. NS5A) according to the standard methods, such as enzyme linked immunosorbent assay (ELISA). Briefly, following incubation with the primary antibody, the plates are washed to remove unbound antibody and then incubated with a second, enzyme-conjugated antibody that can bind to the primary antibody. The plates are washed again, followed by an incubation with a colorless substrate that upon hydrolysis (cleavage) by the enzyme yields a colored product, the concentration of which can be determined with a spectrophotometer (microtiter plate reader). The concentration of the product corresponds to the levels of viral replication in cells and can be used to determine the activity of a given drug to inhibit HCV replication.

## **B. Cytotoxicity Assays**

A cytotoxicity assay may be conducted to evaluate potential anti-HCV agents, utilizing a protocol similar to that described above. Instead of measuring HCV replication levels, however, cytotoxicity of the various test agents is assessed by standard procedures to determine cell viability, proliferation and levels of cellular metabolism including but not restricted to cell membrane permeability, lysosomal mass-pH, cell density or mitochondrial activity. For example, the CytoTox-ONE™ Assay from Promega is a rapid, fluorescent measure of the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into resorufin. Since the CytoTox-ONE™ Reagent mix does not damage

healthy cells, released LDH can be measured directly in assay wells containing a mixed population of viable and damaged cells.

5 **IV. Identification of Cell Lines Permissive for HCV Infection**

As shown herein, it is possible to produce HCV carrying adaptive mutations that confer broad tissue and species tropism. Using such virus stocks it will be possible to screen cell lines of human and non-human origin for virus infection. Briefly, probes which correspond to unique portions of the sequence, may be used in detection methods. This method will lead to the identification of novel cell lines that are permissive for a complete cycle of HCV replication.

**V. Screening for the HCV Receptor(s)**

The ability to replicate HCV in different cell lines facilitates the isolation of the HCV receptor. Virus stocks similar to the ones described in section IV can be used to isolate the HCV receptor(s). For this purpose virus stocks carrying replicons with a selectable marker, such as neomycin or hygromycin will be used. Cells that are non-permissive for infection, will be transfected with DNA isolated from cells that are known to express the receptor (i.e. human hepatocytes, cells identified with the procedure described in section III) and subsequently infected with recombinant HCV carrying the selectable marker. Cells that express the receptor can then be selected through the addition of an antibiotic (i.e. G418 or hygromycin) to the culture medium. Once cells are identified, the transfected DNA can be isolated, cloned, and sequenced. The sequence information can then be used to identify the gene(s) encoded by transfected DNA.



The following examples are provided to describe the invention in further detail. These examples are intended to illustrate and not to limit the invention.

5

**EXAMPLE I Human Non-Hepatic and Mouse Hepatic Cell Lines  
that replicate HCV**

**MATERIALS AND METHODS**

10 Cell culture. Cells were purchased from the American Type Culture Collection (BHK Kidney *Mesocricetus auratus* (Syrian golden hamster) ATCC CRL-1632; Vero Kidney epithelial *Cercopithecus aethiops* (African green monkey) ATCC CCL-81; CV-1 Kidney fibroblast *Cercopithecus*  
15 *aethiops* (African green monkey) ATCC CCL-70; HT1080 Fibrosarcoma *Homo sapiens* (human) ATCC CRL12012; HeLa Cervix carcinoma *Homo sapiens* (human) ATCC CCL2; McA-RH7777 Hepatoma *Rattus norvegicus* (rat) ATCC CRL-1601; FTO2B Hepatoma *Rattus norvegicus* (rat); Hepal-6 Hepatoma  
20 *Mus musculus* (mouse) ATCC CRL-1830; AML12 Hepatocyte *Mus musculus* (mouse) ATCC CRL-2254; FL83B Hepatocyte *Mus musculus* (mouse) ATCC CRL-2390). The Huh7-derived cell lines GS4.1 and GS4.5 are subclones derived from cell lines FCA1 and FCA4, respectively (Guo, J. T., et al.,  
25 2001., J. Virol. 75:8516-8523). Cell line Bsp8 is a Huh7-derived cell line expressing HCV-N subgenomic replicon 1bneoΔS (Guo, J. T., et al., 2001., J. Virol. 75:8516-8523). All cultures were grown in Dulbecco's modified Eagle's medium (Gibco-Invitrogen) supplemented with 10%  
30 fetal bovine serum, L-glutamine, nonessential amino acids, penicillin, and streptomycin.

RNA transfection. All the plasmids were linearized with ScaI, and RNA was synthesized with the MEGAscript kit  
35 (Ambion). In vitro-transcribed RNA was purified as

previously described (Guo, J. T., et al., 2001., J. Virol. 75:8516-8523). Total cellular RNA was extracted with Trizol reagent (Invitrogen). The conditions used for the transfection of cells with total RNA were identical to those used for the transfection with in vitro-transcribed RNA (Guo, J. T., et al., 2001., J. Virol. 75:8516-8523). Colonies were selected with G418 at a concentration of 1 mg/ml.

10 RNA analysis. Total cellular RNA was extracted from transfected cell lines with Trizol reagent. Five micrograms of total RNA was fractionated on 1% agarose gels containing 2.2 M formaldehyde and transferred onto a nylon membrane. Membranes were hybridized with riboprobes specific for plus-stranded HCV replicon RNA, human papillomavirus (HPV) E6, and mouse albumin mRNA as described previously (Guo, J. T., et al., 2001., J. Virol. 75:8516-8523). The HPV and mouse albumin probes spanned nucleotides 811 to 1491 (GenBank accession number M20325) and nucleotides 1501 to 1988 (GenBank accession number XM\_132149), respectively.

Reverse transcription-PCR and DNA sequencing. Nucleotide and amino acid numbers correspond to the HCV type 1b genome Con-1 (AJ238799). HCV replicons were isolated and cloned from established cell lines by PCR amplification of three fragments spanning the entire NS region from position 3420 to 9410. The untranslated regions at the 5' and 3' ends of HCV RNA were cloned separately for nucleotide sequence analysis. DNA synthesis was carried out with Superscript II reverse transcriptase provided in a cDNA synthesis kit (Gibco- Invitrogen). The DNA oligomers used as primers for the reverse transcription reaction mapped to positions 485 to 465, 5492 to 5473, 7256 to 7234, 9410 to 9388, and 9616 to 9597. The

reaction mixtures were incubated for 1 h at 45°C. PCR was performed with an Advantage PCR kit (Clontech). One microliter of the cDNA reaction mixture was used for PCRs with 19- to 23-nucleotide-long primers that yielded  
5 fragments spanning positions 1 to 464, 1387E to 5082, 5016 to 7226, 7154 to 9387, and 9239 to 9616. Position 1387E refers to an oligomer specific for the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) element located upstream of NS3. The PCR  
10 products were cloned into plasmid pGEM-T Easy (Promega). Four clones of each fragment were sequenced with an ABI automatic DNA sequencer, and a consensus sequence was established with the help of a sequence assembly program (Genetics Computer Group).

15 Long reverse transcription-PCR was performed with an Advantage-GC kit (Clontech) with a pair of primers beginning at positions 1415E, upstream of NS3, and 7989 within NS5B. The PCR conditions were modified as follows: step 1, 95°C for 3 min; step 2, 5 cycles, 30 s at 95°C  
20 and 6 min at 72°C; step 3, 27 cycles, 30 s at 95°C and 6 min at 68°C; step 4, 68°C for 6 min. PCR products were gel purified and digested with HindIII and MfeI and replaced with the corresponding fragment in plasmid I377/NS3-3'.

25 Plasmid construction. All plasmids (Table 3) were derived from the parental HCV Con-1 replicon I377/NS3-3' (AJ242652). Subgenomes containing consensus mutations were constructed by replacing DNA restriction fragments  
30 with the corresponding fragments from the pGEM-T Easy cDNA libraries (see above). The resulting plasmids with the amino acid changes in the NS region are listed in Table 3.

35 Immunofluorescence. Cells were plated on coverslips in

six-well plates at least 16 h before treatment, washed with phosphate-buffered saline, and fixed with cold methanol-acetone (1:1) for 15 to 20 min. Next, the cells were blocked in phosphate-buffered saline containing 10% fetal bovine serum for 30 min at room temperature and then incubated with anti-NS5A antibodies (a gift from Chen Liu) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin antibodies (Jackson Laboratories). In addition, cells were stained with the DNA binding fluorochrome DAPI (4',6'-diamidino-2-phenylindole). Coverslips were mounted with antifade agent (Molecular Probes), examined with a Nikon immunofluorescence microscope, and photographed with a charge-coupled device camera.

15

## RESULTS

### HCV replication in cells of nonhepatic origin.

HCV exhibits a very narrow host range and infects only humans and chimpanzees. We question whether this limitation was due to determinants of RNA replication. Because efficient replication of subgenomes depends on genetic adaptations of the replicon (Blight, K. J., et al. 2000. Science 290:1972-1975; Guo, J. T., et al., 2001., J. Virol. 75:8516-8523; Lohmann, V., et al., 2001. J. Virol. 75:1437-1449), presumably to compensate for subtle variations in the cellular environments among cells from different tissues, it was hypothesized that replication in cells of nonhepatic origin would require additional, cell-type-specific adaptive mutations.

Transfection of several primate- and rodent-derived cell lines with subgenomic RNA transcribed from plasmid DNA carrying previously identified adaptive mutations in Huh7 cells did not yield cell lines expressing replicons. To increase the chance for the selection of RNA subgenomes capable of replicating in cells of nonhepatic origin,

subgenomic RNA isolated from Huh7 cell lines that replicate HCV RNA was used. Because of the high rate of nucleotide incorporation errors that occur during RNA-directed RNA synthesis, this population of viral  
5 subgenomes exhibited much greater genetic heterogeneity than did RNA transcribed from a DNA template in vitro.

Upon transfection of HeLa cells with total RNA obtained from Huh7 cell lines GS4.1, GS4.5, and Bsp8, G418-resistant cell clones were obtained. The number of  
10 clones ranged from approximately 2 (Bsp8) to 50 (GS4.1) per 10 µg of total RNA depending on the origin of the RNA used for the transfections. Replicons in these three Huh7-derived cell lines contained different adaptive mutations and replicated two different HCV 1b genomes  
15 (Guo, J. T., et al., 2001., J. Virol. 75:8516-8523). Several HeLa-derived colonies obtained with total RNA from GS4.1 cells were subsequently expanded into seven stable cell lines (SL1 to SL7; Fig. 1A, lanes 4 and 8 to 12). The amounts of viral RNA present in early passages  
20 of these cell lines examined ranged from 0.05 to 7.5 ng/10 µg of total RNA, which corresponded to 20 to 3,000 copies of RNA per cell. In general, the amounts of RNA increased upon passage of cells and reached levels that were comparable to those obtained with the most  
25 productive Huh7- derived cell lines such as GS4.1 (lanes 2 and 4 to 6). As expected, expression of viral gene products could be confirmed by immunofluorescence with antibodies directed against NS5A (Fig. 1B). As with GS4.1 cells, more than 90% of SL1 cells expressed viral  
30 proteins. However, in contrast to Huh7 cell lines where the accumulation of HCV RNA declines approximately 100-fold when cells become confluent, viral replication in HeLa cells was not affected by the growth conditions of the cells, i.e., SL1 cells continued to produce high  
35 amounts of viral RNA even when they became confluent

(results not shown) (Guo, J. T., et al., 2001., J. Virol. 75:8516-8523; Pietschmann, T., et al. 2001. J. Virol. 75:1252-1264).

## 5 Adaptation of HCV replicons.

To determine whether HCV replication in HeLa cells led to the selection of subgenomes with cell-type-specific adaptive mutations, the efficiency by which G418-resistant colonies formed in Huh7 and HeLa cells transfected with total RNA isolated from GS4.1 and SL1 cells was compared. Total RNA from GS4.1 cells led to the selection of approximately 166 G418-resistant colonies per ng of viral RNA in Huh7 cells compared with only 4 colonies in HeLa cells (Table 1). In contrast, total RNA from SL1 cells yielded 160 colonies in HeLa cells compared with about 20 in Huh7 cells. These results indicated that replication in HeLa cells led to the selection of variants with cell-type-specific adaptive mutations that were responsible for the 40-fold increase in colony formation efficiency between amplified RNA in GS4.1 and SL1 cells. Nucleotide sequence analysis of HCV cDNA clones obtained from the SL1 and SL2 cell lines confirmed this view. These data showed that replicons in the two HeLa cell lines maintained the previously identified adaptive mutations in GS4.1 cells and acquired several additional mutations that resulted in amino acid changes in the NS region (Figure 2 and Table 2). Notably, some of the new mutations formed clusters in the NS4B and NS5A regions. In the case of SL1 cells, a deletion of 43 amino acids near the C terminus of NS5A was observed. Of particular interest were mutations in the amino-terminal region of NS4B, because they have so far not been found in cDNAs from replicons in Huh7 cells and hence could have been responsible for the observed adaptation of replicating RNA (Blight, K. J., et al. 2000. Science

290:1972-1975; Guo, J. T., et al., 2001., J. Virol.

75:8516-8523; Krieger, N., et al., 2001. J. Virol.

75:4614-4624; Lohmann, V., et al., 2001. J. Virol.

75:1437-1449). Moreover, one mutation at position 1749

5 was present in both SL1 and SL2 cells. In contrast to the results obtained with the NS regions, no mutations were detected in the 5' and 3' untranslated regions of replicons expressed in SL1 and SL2 cells.

10

**Table 1. Colony formation efficiency of total cellular RNA<sup>a</sup>.**

	No. of colonies in transfected cells							
			Huh7		HeLa		Hepal-6	
	Cell	Viral RNA (ng/10 µg)	Mean (SD)	Colonies/ ng of viral RNA	Mean (SD)	Colonies/ ng of viral RNA	Mean (SD)	Colonies/ ng of viral RNA
15	GS4.1	5	834 (64)	166	22 (4)	4	0	<1
20	SL1	5	100 (53)	20	803 (81)	160	1.3 (1.5)	<1
25	MH1	0.5	20 (2)	40	66 (9)	132	1.7 (0.6)	3

<sup>a</sup> Results from three independent transfection experiments. Total RNA was extracted from GS4.1, SL1, and MH1 cells at passages 21, 26, and 4, respectively.

30

35

40

45

**TABLE 2. Consensus mutations in replicons isolated from HeLa and mouse hepatoma cell clones**

	Cell clone	Conserved mutation(s)	NS protein
5			
	GS4.1	E1202G	NS3
		S2204I, D2254E, I2324V	NS5A
	SL1	Q1067R, S1128A, E1202G, S1323P, S1560G <sup>a</sup>	NS3
		L1701F	NS4A
10		Q1720R, Q1727H, V1749A, V1893L	NS4B
		T2035A, S2204I, I2252V, D2254E, I2274V, R2290L, I2324V, del.2371-2413 <sup>b</sup>	NS5A
		W2990R	NS5B
15	SL2	I1097V, Q1112R, P1115L, V1593M, M1647I	NS3
		L1715P, Q1737R, V1749A, I1797V, N1965Y	NS4B
		Q2012L, S2204I, E2247G, D2254E, K2302R, I2324V, S2336P, L2400S, E2411G, A2412V	NS5A
	MH1	Q1067R, S1128A, E1202G, S1323P, S1560G	NS3
		Q1720R, Q1727H, V1749A, V1893L	NS4B
20		T2035A, S2204I, I2252V, D2254E, I2274V, R2290L, I2324V, M2388T, T2496A	NS5A
		W2990R	NS5B
	MH2	Q1112R, E1202G, <sup>a</sup> S1323P, S1560G	NS3
		L1701F	NS4A
25		Q1720R, Q1727H, V1749A, V1893L	NS4B
		T2035A, T2185A, S2204I, I2252V, D2254E, I2274V, R2290 <sup>a</sup> , I2324V <sup>a</sup>	NS5A
		W2990R <sup>a</sup>	NS5B
	MH4	Q1067R, S1128A, E1202G, S1323P, S1560G	NS3
30		L1701F	NS4A
		Q1720R, Q1727H, V1749A, V1893L, A1841T	NS4B
		T2035A, S2204I, I2252V, D2254E, I2274V, R2290L, I2324V, T2364M, L2391R	NS5A
		I2843V, W2990R	NS5B
35			

<sup>a</sup> Mutation that occurred in 50% of the clones analyzed.

<sup>b</sup> del., deletion.

Mouse hepatoma cells can support HCV RNA replication.

40 The discovery of several additional mutations in cDNA clones obtained from SL1 and SL2 cells suggested total RNA from these cell lines might yield colonies in cells that did not appear to be permissive for HCV replication after transfection with subgenomic RNA or

45 total RNA from Huh7-derived cell lines. Hepatoma and hepatocyte-derived cell lines were examined. G418-



resistant colonies were obtained with the mouse hepatoma cell line Hepa1-6 after transfection with total RNA from SL1 cells (Figure 3A, lanes 4 to 6, 9, and 12). As with HeLa cells, the amounts of RNA ranged from 300 to 1,000  
5 copies of RNA per cell and a large fraction of the cells expressed viral proteins (Figure 3B). In contrast to Huh7 and HeLa cells, the amount of HCV RNA in the mouse cell lines appeared to vary between cell passages (Figure 3A, lanes 6 to 14). Interestingly, total RNA isolated from  
10 one of the mouse cell lines, MH1, did not produce significantly more colonies in Hepa1-6 cells than did total RNA from SL1 cells, suggesting that the subgenomes present in SL1 cells were already adapted for replication in the mouse cells (Table 1). In support of this  
15 interpretation, nucleotide sequence analysis of viral cDNAs cloned from three mouse cell lines showed that the majority of the mutations identified in SL1 cells were maintained (Figure 2). Surprisingly, the deletion in NS5A identified in four of four clones sequenced from SL1  
20 cells was not present in replicons isolated from mouse cells, indicating that a subpopulation of replicons without the deletion was still present in these (SL1) cells.

In further experiments, mouse hepatocyte cells AML12  
25 (ATCC CRL-2254) were transfected with total RNA isolated from the cell line GS4.1, expressing subgenomic replicons and from cell line HFL expressing full-length HCV genomes, respectively. G418 resistant colonies were isolated to establish stable cell lines expressing HCV  
30 suggenomic and full-length replicons. 5 micrograms of total RNA was isolated from AML12 cell lines (MA6-5 to MA6-8, and MAC1) that were established from G418 resistant cell colonies and analyzed by Northern blot analysis, which confirmed replication of HCV (Figure 4).

35

Cell-derived HCV RNA is more efficient than in vitro-transcribed RNA in initiating replication in HeLa and mouse hepatoma cells.

Results showed that replication of HCV subgenomes in HeLa and mouse cells led to the selection of replicons with several novel mutations. The majority of these mutations were located in the NS3, NS4B, and NS5A regions. Moreover, the results showed that cell-derived RNA carrying some or all of these mutations was much more efficient in establishing G418-resistant colonies in HeLa cells than was RNA derived from Huh7 cells (Table 1).

Based on these observations, it was surmised that introduction of these mutations into available subgenomic replicons should alter or expand their tissue and host tropism. To test this hypothesis, 13 subgenomic replicons were designed that carried mutations in NS3, NS4B, and NS5A alone or in combination with each other as described in Table 3. Of the 13 constructs examined, only two, pZS2 and pZS25, yielded a small number of G418-resistant colonies in HeLa cells (Table 4). Viral RNA replication was confirmed by Northern blot analysis of total RNA isolated from six cell lines derived from those colonies. None of the variants yielded colonies in Hepa1-6 cells. Moreover, negative-control experiments with in vitro-transcribed RNA derived from a variant containing a frameshift mutation in NS5B did not yield any colonies that could be expanded into cell lines. Notably, save for one, all replicons were permissive for replication in Huh7 cells, albeit with significantly different efficiencies (Table 3). Interestingly, both pZS2 and pZS25 carried mutations in NS4B that were conserved in replicons from two independent HeLa cell lines, SL1 and SL2. In addition, these replicons had the S2204I mutation in NS5A that was previously found to be one of the most potent adaptive mutations for HCV replication in Huh7

cells. Because both replicons replicated very efficiently in Huh7 cells, the results suggested that the NS4B mutations could have contributed to the observed expansion of the tissue tropism of HCV replicons. In support of this hypothesis, the subgenome with the highest efficiency in Huh7 cells, pZS11 lacking mutations in NS4B (Table 3), did not yield any colonies in HeLa cells.

**TABLE 3. Colony formation efficiency of mutant replicons in Huh7 cells**

Vector <sup>1</sup>	Conserved mutation(s)	Mean (SD) CFE/ µg of RNA <sup>g</sup>	NS protein(s) affected
I <sub>377</sub> /NS3-3'	NA <sup>b</sup>	2.3 (1.5)	NA
pZS10	A1 <sup>a</sup>	3.3 (3.5)	NS3
pZS1	C1 <sup>b</sup>	1.4 X 10 <sup>3</sup> (3.9 X 10 <sup>2</sup> )	NS5A
pZS20	B <sup>c</sup>	1.7 (0.6)	NS4AB
pZS11	A1 + C1	2.4 X 10 <sup>5</sup> (7 X 10 <sup>4</sup> )	NS3, NS5A
pZS2	B + C1	7.8 X 10 <sup>4</sup> (1.7 X 10 <sup>4</sup> )	NS4AB, NS5A
pZS12	A1 + B	0.3 (0.6)	NS3, NS4AB
pZS5	A1 + B + C1	15	NS3, NS4AB, NS5A
pZS4	B + C1 + A2 <sup>d</sup>	165 (21)	NS3, NS4AB, NS5A
pZS8	A2 + B	0	NS3, NS4AB
pZS6	A2 + B + C2 <sup>e</sup>	8 (4)	NS3, NS4AB, NS5A
pZS15	C3 <sup>f</sup>	491 (183)	NS5A
pZS25	B + C3	1.7 X 10 <sup>4</sup> (2.6 X 10 <sup>3</sup> )	NS4AB, NS5A
pZS45	A2 + B + C3	19 (2)	NS3, NS4AB, NS5A

<sup>a</sup> A1, mutation E1202G.

<sup>b</sup> C1, mutations S2204I and D2254E.

<sup>c</sup> B, mutations L1701F, Q1720R, Q1727H, and V1749A.

<sup>d</sup> A2, mutations E1202G, S1128A, and S1323P.

<sup>e</sup> C2, mutations S2204I, D2254E, R2290I, and I2324R.

<sup>f</sup> C3, all the mutations of C2 plus the deletion 2371-2413.

<sup>g</sup> CFE, colony formation efficiency. Values are derived from three independent transfections of each replicon RNA.

<sup>h</sup> NA, not applicable.

<sup>1</sup> Sequence ID Numbers for subgenomic replicons are listed in Table 6

**TABLE 4. Colony formation efficiency of in vitro-transcribed RNA<sup>a</sup>**

5	Source of cDNA library (cell line or plasmid)	No. of colonies in transfected cells		
		Huh7	HeLa	Hepal-6
10	GS4.1	>10,000	0, 0, 0	0, 1, 0
	SL1	>10,000	0, 3, 2	0, 1, 0
	MH4	>10,000	3, 4, 0	17, 0, 0
	pZS2	>10,000	2, 3, 0	0, 0, 0
	pZS25	>10,000	0, 2, 1	0, 0, 0

15       <sup>a</sup> Results from transfection experiments with in vitro-transcribed RNA from pooled clones isolated from the indicated cell line and from in vitro-transcribed RNA from pZS2 and pZS25 (Table 3).

20       To further explore the basis for the observed low colony formation efficiency of in vitro-transcribed RNA in HeLa cells, it was determined if replication in HeLa cells led to the selection of adaptive mutations that were not discovered previously when cDNA clones from SL1 and SL2 cells were sequenced. For this purpose, cDNA clones were isolated from total RNA obtained with pZS2- and pZS25-derived cell lines, respectively. Nucleotide sequence analysis of both cDNA clones did not reveal any additional consensus mutations, suggesting that the two subgenomes were sufficiently adapted for replication in HeLa cells (results not shown). However, as mentioned above, it was possible that a minor population of subgenomic replicons with additional mutations were present in these cell lines. To overcome this problem, a method for the isolation and cloning of cDNAs spanning the NS3 to NS5B region was developed (see Materials and Methods). Replicon cDNA libraries were produced from GS4.1, SL1, and MH4 cells. Approximately 2,000 cDNA

clones were pooled and subsequently used for in vitro transcription of subgenomic RNA. With Huh7 cells, the colony formation efficiency of the pooled clones was comparable to that of the most efficient subgenomes, such as pZS2 or pZS25, and did not vary significantly with the origin of the total RNA used for cDNA cloning (Table 4). Consistent with previous results, colony formation in HeLa and mouse cells was origin dependent, i.e., save for one case, colonies were observed only with clones derived from SL1 and MH4 cell lines. Notably, with this strategy G418-resistant colonies were obtained for the first time with Hepal-6 cells by using in vitro-transcribed RNA. To confirm the presence of viral RNA, 11 colonies were expanded and Northern blot analysis was performed with total RNA. All 11 RNA samples analyzed contained viral RNA ranging from approximately 0.1 to 1 ng/5 µg of total RNA (results not shown).

Taken together, the results supported the hypothesis that mutations identified in subgenomic replicons expressed in HeLa and mouse cells play a role in adaptation of the replicons to certain cell-type-specific conditions.

## DISCUSSION

HCV is known as a species- and tissue-specific virus. The results described herein show that replication of HCV can occur in cells derived from tissues other than liver, indicating that cellular factors required for RNA replication are expressed in cell types other than hepatocytes. One interpretation of this result is that the apparent tropism of HCV for hepatocytes is determined primarily at the level of virus entry or assembly or, alternatively, that HCV can infect many other tissues but has escaped detection due to very low amounts of RNA replication or accumulation. Extrahepatic tissues could

serve as reservoirs for HCV that, as with human immunodeficiency virus, could provide a source of viruses that are refractory to antiviral therapy and, importantly, can be responsible for infection of liver  
5 grafts following orthotopic liver transplantation (Chun, T. W., et al., 2002. J. Infect. Dis. 185:1672-1676; Laskus, T., et al., 2002. J. Infect. Dis. 185:417-421). Such a scenario would have profound implications for antiviral therapy. For example, the targeting of drugs to  
10 secondary sites of viral replication and the analysis of drug metabolism in cells other than hepatocytes would become important factors for the development of successful antiviral therapies.

It is conceivable that HCV quasispecies in  
15 hepatocytes and other tissues exhibit differences in their composition due to the selection of variants with cell-type-specific adaptations. As shown in this Example, replication of subgenomes in HeLa cells led to the accumulation of clusters of mutations in the NS3, NS4B, and NS5A regions including a deletion in NS5A (Figure 2). Mutations and deletions in NS5A have been found  
20 previously in genomes that replicated in Huh7 cells, which could suggest that expression of the natural form of this protein in cell culture somehow interferes with RNA replication (Blight, K. J., et al. 2000. Science  
25 290:1972-1975; Guo, J. T., et al., 2001., J. Virol. 75:8516-8523; Ikeda, M., et al., 2002. J. Virol. 76:2997-3006; Lohmann, V., et al., 2003. J. Virol. 77:3007-3019; Lohmann, V., et al., 2001. J. Virol. 75:1437-1449).  
30 However, mutations in the amino terminus of NS4B have previously not been observed. Notably, in both SL1 and SL2 cells, the mutations changed two or one glutamine residues, respectively, to one of the two basic amino acids arginine and histidine. Moreover, the mutation  
35 V1749A was present in all five cell lines examined (Table

2 and Figure 2). Thus far, these results show that these mutations appear to be required for replication in HeLa cells, because only replicons pZS2 and pZS25 carrying these mutations yielded colonies after transfection with  
5 in vitro-transcribed RNA (Tables 3 and 4). The amino terminus of NS4B is predicted to reside on the cytoplasmic side of endoplasmic reticulum membranes and may interact with other host or viral proteins required for RNA replication (Hugle, T., et al., 2001. Virology  
10 284:70-81). As an integral endoplasmic reticulum membrane protein, NS4B might provide a scaffold for the assembly of replication complexes and act as a regulator for RNA replication. More importantly, a recent study revealed that NS4B can induce particular membrane structures,  
15 called membranous webs, proposed to be the site for HCV replication (Egger, D., et al., 2002. J. Virol. 76:5974-5984). Interestingly, genetic analyses with an HCV-related pestivirus identified the amino-terminal region of NS4B as a determinant for cytotoxicity caused by high  
20 levels of virus replication (Qu, L., et al., 2001. J. Virol. 75:10651-10662).

In summary, this example demonstrates that HCV RNA replication is not restricted to the human hepatoma cell line Huh7 but instead occurs in HeLa cells and hepatoma  
25 cells derived from mice. These findings further facilitate development of a mouse model for HCV infection.

#### **Example II RNA Polymerase Inhibitors Inhibit HCV** 30 **replication in Transformed HeLa Cells**

In this example, the anti-HCV activity of 2'-C-methyladenosine (2CMA, an HCV RNA polymerase inhibitor) was tested on GS4.1 (Huh7) cells, and SL1 (HeLa). The  
35 cells were treated with 10  $\mu$ M 2CMA. The cells were

harvested at 6, 12, 24, 48, and 72 hours. Total cellular RNA was extracted and viral RNA (vRNA) analyzed by Northern blot analysis. These results indicate that 2CMA effectively inhibits HCV in GS4.1 and SL1 cells (Figure 5).

Next, the antiviral activity of the HCV RNA polymerase inhibitor 5-OH-cytidine was tested. GS4.1 (Huh7) cells and SL1 (HeLa cells were treated with the indicated amounts of 5-OH-cytidine. The DNA polymerase inhibitor 5-OH-deoxy-cytidine was used as a negative control. The cells were harvested 72 hours after incubation with the drugs. Total cellular RNA was extracted and viral RNA analyzed by Northern blot analysis. The intensity of the bands corresponding to HCV RNA was determined with a Fuji phosphoimager. These results indicate that 5-OH-cytidine effectively inhibits HCV replication in GS4.1 and SL1 cells (Figure 6).

**Example III Cytopathic and NonCytoPathic Responses**  
**in Cells Expressing Hepatitis C Virus**

Currently, combination treatment with alpha-interferon and ribavirin is the therapy of choice for HCV infection. But this treatment is not always effective, and other treatment choices are limited, or have unproven efficacy. Study of the mechanism of action of IFN- $\alpha$  may help elucidate a new, effective treatment for HCV, or help determine what makes HCV treatment effective.

DNA microarray studies revealed that the antiviral response induced by IFNs alters the expression of hundreds of genes and, hence, is far more complex than previously anticipated (Der, S. D., et al., 1998. Proc. Natl. Acad. Sci. USA 95:15623-15628). Little is known about the nature of the cellular proteins that specifically target viral components and, hence, are responsible for the inhibition of viral replication in



the absence of cell death. In contrast, the major signal transduction pathways required for the innate immune response against many viruses have been elucidated. The first wave of IFN-induced genes depends on the phosphorylation of STAT1 and STAT2 and their interaction with IRF9 (p48) to form the transcription factor complex ISGF3. In addition, viral double stranded RNA (dsRNA) and other unknown viral factors are believed to play an important role in the establishment of an antiviral state. They can activate dsRNA-dependent enzymes such as protein kinase R (PKR) and 2',5'-oligoadenylate synthase (OAS), as well as other still-elusive protein kinases (Smith, E. J., et al., 2001. J. Biol. Chem. 276:8951-8957). IFN- $\alpha$  can induce a noncytopathic antiviral response or, alternatively, trigger apoptotic programs leading to the elimination of infected cells (Tanaka, N., et al., 1998. Genes Cells 3:29-37).

Nucleotide sequence analyses of HCV genomes isolated from Japanese patients revealed a correlation between the presence of mutations in a short segment of NS5A, termed the IFN sensitivity-determining region (ISDR), and resistance to antiviral therapy with IFN- $\alpha$  (Enomoto, N., et al., 1995. J. Clin. Investig. 96:224-230; Enomoto, N., et al., 1996. N. Engl. J. Med. 334:77-81). Subsequently, it was reported that the ISDR motif can bind to PKR (Gale, M. J., Jr., et al., 1997. Virology 230:217-227). Importantly, the ISDR from IFN-resistant, but not from IFN-sensitive, HCV isolates appeared to be a substrate for PKR, suggesting that IFN treatment of chronically infected patients can lead to the selection of HCV variants with ISDRs that can bind and inactivate PKR (Gale, M. J., Jr., et al., 1998. Clin. Diagn. Virol. 10:157-162; Tan, S. L., and M. G. Katze. 2001. Virology 284:1-12).

Accordingly, study of HCV variants and the pathway

by which IFN inhibits HCV is necessary to provide new HCV treatments, and to prevent selection of IFN- $\alpha$  resistant variants.

## 5 MATERIALS AND METHODS

Chemicals and reagents. Recombinant IFN- $\alpha$ 2b (intron A) was purchased from Schering-Plough. Cycloheximide, 2-aminopurine (2-AP), genistein, sodium salicylate, and wortmannin were obtained from Sigma. SB 203580, PD 98059, 10 vanadate, PP2, rapamycin, and lactacystin were obtained from Calbiochem. Epoxomicin was obtained from Boston Biochem, and caspase inhibitor ZVAD- fluoromethyl ketone (ZVAD-FMK) was obtained from Enzyme Systems Products.

15 Cell culture. Huh7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin G, streptomycin, nonessential amino acids, and L-glutamine. For the cell lines carrying HCV and Kunjin virus replicons, 500  $\mu$ g of 20 G418/ml was added to the medium. The GS4.1 cell line was derived from a subclone of FCA1 cells as described previously (Guo, J. T., et al., 2001. J. Virol. 75:8516-8523). SL1 is a HeLa cell line expressing HCV subgenomic replicon I<sub>377</sub>NS3-3' (Lohmann, V., et al., 1999. Science 25 285:110-113; Zhu, Q., et al., 2003. J. Virol. 77:9204-9210). The KUNCD20 cells represent a pool of approximately 200 colonies of G418-resistant HeLa cells obtained after transfection with the Kunjin virus replicon C20DXrepNeo RNA (Khromykh, A. A., and E. G. 30 Westaway. 1997. J. Virol. 71:1497-1505) (kindly provided by A. Khromykh, Sir Albert Sakzewski Virus Research Center, Brisbane, Australia).

Plasmids. pCMV-E3L expressing the vaccinia virus E3L 35 protein was obtained from Robert Schneider, New York

University, New York. pln035 expressing virus-associated (VA) RNA was obtained from David Lazinski, Tufts University, Boston, Mass. pEF-HA-HPIV2 expressing the V protein of human parainfluenzavirus 2 (HPIV2) was  
5 obtained from Curt Horvath, Mount Sinai School of Medicine, New York, N.Y. To obtain cDNA clones of the gene encoding human Mx-1, Huh7 cells were treated with 100 IU of IFN- $\alpha$ /ml for 6 h and total cellular RNA was extracted with TRIzol reagent (Invitrogen) and first-  
10 strand cDNA was made with an oligo(dT)<sub>12-18</sub> primer and Superscript II DNA polymerase (Invitrogen) by following the manufacturer's direction. For the amplification of Mx-A cDNA, the primers used were 5'-  
AGTATCGTGGTAGAGAGCTGC-3' (SEQ ID NO:15) and 5'-  
15 TAATACGACTCACTATAGGGATGTGGCTGGAGATGC-3' (SEQ ID NO:16). The purified PCR fragment was cloned into the pGEM-T Easy vector (Promega). The identity of the cloned fragment was verified by nucleotide sequence analysis.

20 RNA extraction and Northern blot hybridization. Total cellular RNA was extracted with TRIzol reagent (Invitrogen) by following the manufacturer's direction. Five micrograms of total RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde and transferred  
25 onto nylon membranes. Membranes were hybridized with riboprobes specific for plus-stranded HCV replicon RNA and Mx-A and  $\beta$ -actin mRNA in the conditions described previously (Guo, J. T., et al., 2001. J. Virol. 75:8516-8523).

30 Detection of eIF-2 $\alpha$  phosphorylation by Western blotting. For Western blot analysis of eIF-2 $\alpha$  phosphorylation, cells were treated with 100 IU of IFN- $\alpha$ /ml for 12 h and then transfected with 2  $\mu$ g of poly(I:C) per 60-mm-  
35 diameter plate by using Lipofectamine (Invitrogen). After

3 h of incubation, cells were lysed in high-salt radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 250 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). Proteins (40 µg) were  
5 separated on SDS-10% polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Membranes were incubated with 50% methanol, washed extensively with water, and blocked with 3% casein in TNET buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1  
10 mM EDTA, 0.05% Tween 20). Membranes were incubated with rabbit polyclonal antibodies against eIF-2α (a gift from Robert Schneider, New York University) or phosphorylated eIF-2α (eIF-2α-P; Research Genetics, Inc.) diluted in blocking solution for 1 h and then washed extensively  
15 with TNET buffer. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit and immunoglobulin G (IgG) (Amersham), respectively. The bound IgG was detected with Super-Signal chemiluminescence reagents (Pierce).

20

RPA. For the analysis of IFN-β gene expression, cells were treated with 100 IU of IFN-α/ml for 12 h and then transfected with 2 µg of poly(I:C) per 60-mm plate by using Lipofectamine (Invitrogen). After 3 h of  
25 incubation, total cellular RNA was extracted with TRIzol reagent (Invitrogen), and IFN-β mRNA levels were determined by RNase protection assay (RPA) with the help of the RPAII kit purchased from Ambion. The probes complementary to IFN-β (GenBank accession no. M25460) and  
30 β-actin (GenBank accession no. BC013380) mRNAs spanned positions 272 to 650 and 1030 to 1250, respectively.

Annexin V-FITC staining. SL1 cells were plated on coverslips in six-well plates 16 h prior to treatment.  
35 Cells were then mock treated or treated with 100 IU of

IFN- $\alpha$ /ml in the absence or presence of 20  $\mu$ M ZVAD-FMK. Coverslips were then put on slides and incubated with 100  $\mu$ l of staining solution containing annexin V-fluorescein isothiocyanate (FITC) at room temperature for 10 to 15 min. After extensive washes with phosphate-buffered saline, slides were examined with a Nikon fluorescence microscope and photographed with a charge coupled device camera.

10 Flow cytometry analysis. To determine the fraction of apoptotic cells, the annexin V assay system (Roche Diagnostics GmbH) was used. Cells were incubated with IFN- $\alpha$  (100 IU/ml) alone or together with 20  $\mu$ M ZVAD-FMK for 24 h. The culture medium containing detached cells  
15 was collected, and the adherent cells were trypsinized and then combined with the detached cells. The cells were collected by centrifugation and washed once in phosphate-buffered saline. Pelleted cells were resuspended in binding buffer and were incubated with annexin V-FITC at  
20 room temperature for 10 to 15 min. The stained cells were then diluted with binding buffer and analyzed by flow cytometry (FACScan; Becton Dickinson).

## RESULTS

25 IFN- $\alpha$  can induce noncytopathic and cytopathic antiviral responses in cells comprising HCV replicons.

As set forth above, stable HeLa cell lines were established that express HCV subgenomes with an efficiency similar to that of Huh7 cells (Zhu, Q., et al., 2003. J. Virol. 77:9204-9210). Examination of the  
30 IFN- $\alpha$  response in HeLa derived cell lines such as SL1 revealed a very similar dose dependent reduction of virus replication. The IC<sub>50</sub> of IFN- $\alpha$  in HeLa cell lines was generally in the range of 0.1 IU/ml, approximately 10-  
35 fold lower than the IFN- $\alpha$  IC<sub>50</sub> in Huh7-derived cell lines

(Figure 7). However, in marked contrast to observations made with Huh7 cell lines, treatment of SL1 and other HeLa derived cell lines with more than 30 IU of IFN- $\alpha$ /ml induced cell death in a significant fraction of cells between 6 and 20 h post treatment (Figure 8A). Cell death was caused by apoptosis, as determined by annexin V staining, and could be prevented by the caspase inhibitor ZVAD-FMK. The fraction of apoptotic cells was determined before and after treatment with the cytokine. The results showed that IFN- $\alpha$  induced apoptosis in more than 30% of SL1 cells compared with 6 to 7% in untreated SL1 and parental HeLa cells (Figure 8B). Several other HeLa-derived cell lines were examined to assure that the results obtained with SL1 cells reflected a general property of HeLa cells expressing HCV subgenomes. Moreover, to test more directly whether IFN-induced apoptosis was caused by viral replication, two methods were used to inhibit RNA replication in SL1 cells. The first was based on the observation that replication of HCV subgenomes is temperature sensitive and is inhibited at 39°C (J. A. Sohn and C. Seeger, unpublished observations). The second method relied on the availability of an inhibitor of the viral RNA polymerase. Consistent with a role for viral replication in the induction of apoptosis, cell death could be prevented when viral replication was inhibited by either incubation of the cells for 60 h at the elevated temperature or treatment with a viral polymerase inhibitor (Figure 8B and result not shown).

These results raised the question of whether IFN-induced apoptosis reflected a general property of HeLa cells expressing viral replicons. To address this problem, the IFN response against HCV in SL1 cells was compared with that against the flavivirus Kunjin virus in HeLa cells. For this purpose a pool of HeLa cells,

KUNCD20, expressing Kunjin virus subgenomic replicons lacking the structural genes, similar to the HCV subgenomic replicons was established (Khromykh, A. A., and E. G. Westaway. 1997. J. Virol. 71:1497-1505). The  
5 Kunjin virus RNA levels in these cells were approximately fivefold higher than those observed with HCV in SL1 cells. In contrast to results with SL1 cells, treatment of KUNCD20 cells with different concentrations of IFN- $\alpha$  only slightly inhibited viral replication (Figure 9).  
10 Importantly, cell death in IFN- $\alpha$ - treated KUNCD20 cells was not detected either by light microscopy or annexin V staining (results not shown). These results indicated that IFN-induced apoptosis is a property of HCV-expressing HeLa cells rather than a general property of  
15 HeLa cells replicating viral RNA genomes.

In summary, these results demonstrated that IFN- $\alpha$  could induce noncytopathic as well as cytopathic antiviral programs in cells expressing HCV replicons in a concentration- and cell type-dependent fashion. Moreover,  
20 the results showed that this antiviral program was specific for HCV replicons. Importantly, the results suggested that HCV replication could induce an innate cellular response that, in combination with IFN- $\alpha$ , could lead to apoptosis.

25

IFN- $\alpha$  inhibits HCV replication through the Jak-STAT signal transduction pathway

Information about the signal transduction pathways responsible for execution of the IFN response has  
30 generally been obtained with cells treated with high concentrations (100 to 1,000 IU/ml) of the cytokine and with fibroblasts and epithelial cells, most of which cannot, to date, support HCV replication. Moreover, a recent study by Schlaak, et al. (Schlaak, J. F., et al.,  
35 2002., J. Biol. Chem. 277:49428-49437) revealed that the

IFN response could vary in a cell type-dependent manner. In addition, it was found that slight changes in cell culture conditions had major effects on HCV replication. Therefore, the observation that replication of HCV in  
5 both Huh7 and HeLa cells could be inhibited with low concentrations of the cytokine warranted a more careful study of the pathways involved in the antiviral program against HCV.

To investigate the nature of the IFN- $\alpha$  response  
10 against the HCV replicon, drugs that were known to inhibit specific components of selected signal transduction pathways that play a role in the antiviral response induced by IFN- $\alpha$  were used (Table 5). The current model for the signal transduction pathway induced  
15 by IFN- $\alpha$  predicts that the IFN receptor associated tyrosine kinases Jak1 and Tyk2 are activated and, in turn, phosphorylate the transcription factors STAT1 and STAT2, which are required for the induction of the cellular antiviral program (Figure 10) (Sen, G. C. 2001.,  
20 Annu. Rev. Microbiol. 55:255-281; Stark, G. R., et al., 1998. Annu. Rev. Biochem. 67:227-264). Incubation of GS4.1 cells with the tyrosine kinase inhibitor genistein suppressed the induction of the IFN-induced Mx-1 gene (Figure 11A and 11B). Similarly, genistein antagonized  
25 the IFN- $\alpha$  response against the HCV replicon. An increase in the concentration of the drug from 100 to 300  $\mu$ M led to a complete inhibition of the IFN response against HCV (results not shown). Consistent with this result, it was found that the V protein of HPIV2 blocked the IFN  
30 response. The V protein of HPIV2 induces the degradation of STAT2 and, hence, inhibits the IFN-induced activation of gene expression (Parisien, J. P., et al., 2002. J. Virol. 76:4190-4198) (Figures 11C and 11D). IFN- $\alpha$  treatment of cells expressing HPIV2 led to a twofold  
35 reduction of viral RNA levels. When adjusted for the



observed transfection efficiency, i.e., 40 to 45% of the cells express the V protein, the reduction corresponded to a complete inhibition of the IFN- $\alpha$  response. Finally, the decline of viral RNA levels was reduced in GS4.1  
5 cells with IFN- $\alpha$  and cycloheximide, indicating that de novo protein synthesis was required for an antiviral response against HCV replication (Table 5).

IFN- $\alpha$  can activate, in addition to the STAT pathway, MAPKs, including extracellular signal-regulated kinase, p38 MAPK, and phosphatidylinositol 3 (PI3)-kinase-Akt  
10 pathways (David, M., et al., 1995. Science 269:1721-1723; Goh, K. C., et al., 1999. EMBO J. 18:5601-5608; Pfeffer, L. M., et al., 1997. Science 276:1418-1420). However, in contrast to genistein, SB 203580, sodium salicylate, and  
15 wortmannin, known inhibitors of p38 MAPK, NF- $\kappa$ B, and PI-3 kinase, respectively, did not inhibit the IFN response at detectable levels, suggesting that the two major ancillary signaling pathways activated by IFN- $\alpha$  were not directly involved in inhibiting HCV replication in Huh7  
20 cells (Table 5; results not shown).

In summary, the results showed that inhibition of HCV replication with IFN- $\alpha$  depended on a functional Jak-STAT pathway (Figure 10). Hence, the results demonstrated that the IFN response against HCV was genuine and did not  
25 reflect an unspecific effect of the cytokine.

Does HCV replication induce an antiviral state in infected cells? A critical step in activation of innate immunity is the induction of an antiviral state by dsRNA  
30 or viral proteins (Figure 10) (Taniguchi, T., and A. Takaoka. 2002. Curr. Opin. Immunol. 14:111-116; tenOever, B. R., et al., 2002. J. Virol. 76:3659-3669). As shown above, evidence for such a virus-induced activation was also obtained from IFN-treated HeLa cells expressing HCV  
35 replicons. To investigate the nature of this HCV-induced

activation, the phosphorylation levels of eIF-2 $\alpha$  and expression of IFN- $\beta$  were determined. eIF-2 $\alpha$  is a substrate of PKR, which is activated by dsRNA that can accumulate as a consequence of viral RNA replication (Srivastava, S. P., et al., 1998. J. Biol. Chem. 273:2416-2423; Williams, B. R. 3 July 2001, posting date. Signal integration via PKR. Sci STKE 2001:RE2. [Online.]). IFN- $\beta$  gene transcription is activated through the coordinate actions of three families of transcription factors NF- $\kappa$ B, IRF3, and ATF2, all of which are activated by dsRNA and/or certain viral proteins (Figure 10) (Peters, K. L., et al., 2002. Proc. Natl. Acad. Sci. USA 99:6322-6327; tenOever, B. R., et al., 2002. J. Virol. 76:3659-3669).

First the levels of eIF-2 $\alpha$ -P in Huh7, GS4.1, HeLa, and SL1 cells was determined in the presence and absence of dsRNA and IFN- $\alpha$ . The results showed that eIF-2 $\alpha$ -P levels were not significantly elevated in cells expressing HCV replicons (GS4.1 and SL1) compared with those in their parental cells (Huh7 and HeLa) (Figure 12A). Similarly, incubation of cells with IFN- $\alpha$  did not induce eIF-2 $\alpha$ -P levels. In contrast, transfection of cells with poly(I:C), mimicking dsRNA, augmented eIF-2 $\alpha$ -P levels, particularly in HeLa and SL1 cells. Similar results were obtained when cells were primed with IFN- $\alpha$  prior to transfection with poly(I:C). These results were confirmed with several other cell lines derived from Huh7 and HeLa cells.

Second, the levels of IFN- $\beta$  mRNA in the four cell lines was determined under the same conditions described above. In agreement with the above results, viral replication alone was not sufficient to activate IFN- $\beta$  gene expression in both Huh7- and HeLa-derived cell lines (Figure 12B). In Huh7 cells and GS4.1 and other Huh7-derived cells expressing HCV replicons, only a weak

induction of IFN- $\beta$  was observed when cells were primed with IFN- $\alpha$  and then transfected with poly(I:C). In contrast, IFN- $\beta$  transcription was induced in HeLa and SL1 cells by poly(I:C) alone and particularly in combination  
5 with IFN- $\alpha$ . Remarkably, expression of IFN- $\beta$  could be induced by IFN- $\alpha$  alone in SL1 cells but not in HeLa cells. Similar results were obtained with the HeLa-derived SL2 cell line (results not shown).

In summary, the results showed that, while both Huh7  
10 and HeLa cells were competent to activate dsRNA-dependent signal transduction pathways, HCV replication alone was not sufficient to induce a detectable dsRNA response in these cells. This result could indicate that dsRNA either does not accumulate during HCV replication or cannot  
15 access PKR and other dsRNA binding proteins. Importantly, the results showed that, despite the apparent lack of biologically active dsRNA, viral replication could activate certain cellular signal transduction pathways that could cooperate with IFN- $\alpha$  to activate the  
20 transcription of the IFN- $\beta$  gene.

The results presented above favored a model predicting that IFN- $\alpha$  inhibited HCV replication by a mechanism that was independent of dsRNA-activated antiviral pathways. To test this model more carefully,  
25 the IFN response was measured in GS4.1 cells expressing the vaccinia virus E3L protein. E3L is known to sequester dsRNA and prevent PKR and OAS/Rnase L activation (Chang, H. W., et al., 1992. Proc. Natl. Acad. Sci. USA 89:4825-4829; Rivas, C., et al., 1998. Virology 243:406-414)  
30 (Figure 10). Indeed, expression of E3L had no measurable effect on the IFN response against HCV (Figure 11C and 11D). Experiments relying on simultaneous detection of E3L and the HCV protein NS5A in the same cell by immunofluorescence confirmed that IFN- $\alpha$  could inhibit HCV  
35 replication in cells expressing the E3L protein (results

not shown). Finally, it was found that the PKR inhibitors 2-amino purine (2-AP) and adenovirus VA RNA did not block the IFN response against HCV in Huh7 cells (Table 5).

**TABLE 5. Effects of inhibitors on the activity of IFN- $\alpha$  against the HCV replicon<sup>a</sup>**

Drug, protein, or RNA	Concn	Primary target(s)	Effect
2-AP	10 mM	PKR and other kinases	-
Genistein	300 $\mu$ M	Tyrosine kinases	+
Cycloheximide	10 $\mu$ g/ml	Translation	+
Sodium salicylate	5 mM	IKK	-
SB 203580	20 $\mu$ M p38	MAPK	-
PD 98059	50 $\mu$ M	MEK kinase	-
Vanadate	50 $\mu$ M	Protein phosphatase	-
Wortmannin	100 nM	PI3 kinase	-
PP2	50 $\mu$ M	src kinase	-
Rapamycin	200 nM	mTOR, translation	-
Lactacystin	5 $\mu$ M 26S	proteasome	+
Epoxomicin	1 $\mu$ M 26S	proteasome	+
V protein of HPIV2		STAT2	+
E3L protein		PKR and OAS	-
VA RNA		PKR	-

<sup>a</sup> GS4.1 cells were incubated with the indicated compounds for 2 h and then the presence of 100 IU of IFN- $\alpha$ /ml for an additional 24 h. Viral RNA levels were determined by Northern blot analysis. Assays for V protein, E3L, and VA RNA are described in the legend to Fig. 5.

What are the pathways that play a role in the IFN- $\alpha$  response against HCV? A major question concerns the mechanism by which IFN- $\alpha$  induces the noncytopathic inhibition of HCV replication. DNA microarray analyses of IFN- $\alpha$ -treated GS4.1 cells and other Huh7-derived cell lines revealed the induction of several classes of genes belonging to known signal transduction and protein degradation pathways (J. Hayashi and C. Seeger, unpublished results; Cheney, I. W., et al. 2002. J. Virol. 76:11148-11154). In particular, several genes encoding proteasome subunits and ubiquitin-like proteins were among the genes most highly induced by IFN- $\alpha$ .

Notably, kinetic studies of HCV replication in Huh7 cells indicated that replication complexes have a relatively short halflife, which is further reduced by IFN treatment (J.-T. Guo and C. Seeger, unpublished observations).

5 Therefore, it was surmised that the proteasome could play a role in inhibition of HCV replication in IFN-treated cells.

To test this hypothesis, the outcome of combination treatment with IFN- $\alpha$  and the proteasome inhibitors  
10 lactacystin and epoxomicin for HCV RNA replication in GS4.1 cells was determined. The cells were pretreated with different concentrations of the inhibitors for 1 h before IFN- $\alpha$  was added for an additional 6 h of combination treatment. Then the cells were incubated for  
15 12 h before RNA was isolated and subjected to Northern blot analysis (Figure 13A). The relatively short incubation period was necessary because of the known toxicity of proteasome inhibitors after longer incubation times. The results showed that HCV RNA levels dropped 70%  
20 within 18 h of IFN- $\alpha$  treatment, whereas in the presence of epoxomicin or lactacystin the reduction was only 30% (Figure 13B). Lower doses of epoxomicin than of lactacystin were effective, which is consistent with the high specific activity of epoxomicin against the  
25 chymotrypsin-like activity of proteasomes (Fenteany, G., and S. L. Schreiber. 1998. J. Biol. Chem. 273:8545-8548; Meng, L., et al., 1999. Proc. Natl. Acad. Sci. USA 96:10403-10408). Treatment with higher doses of lactacystin alone led to a slight reduction of HCV RNA  
30 levels. These results were confirmed with a second set of experiments. The cells were pretreated with 5  $\mu$ M lactacystin and 1  $\mu$ M epoxomicin, respectively, for 1 h before IFN- $\alpha$  was added for an additional 6 h of combination treatment. RNA was isolated from the treated  
35 cells either 6 or 12 h after incubation with IFN- $\alpha$

(Figure 14). The results showed that, at both time points, viral RNA levels were significantly higher in cells that were exposed to the proteasome inhibitors than in cells that were treated with IFN- $\alpha$  alone.

5        Finally, tests were conducted to determine whether proteasome activity was required for induction of the IFN response or, more directly, for inhibition of HCV replication. To distinguish between these two possibilities, first GS4.1 cells were incubated with the  
10        cytokine for 10 h to induce the antiviral response. Then, the cells were incubated for 12 h in the presence of lactacystin or epoxomicin. Under these conditions, the IFN response against HCV remained effective and reduced RNA levels to similar extents independently of the  
15        presence of either of the two inhibitors (Figure 15). Thus, these results indicated that the activity of proteasomes is required for the induction of the IFN response against HCV, but apparently not for direct inhibition of viral replication (see Discussion).

20

## DISCUSSION

      In this Example, the mechanism of the IFN- $\alpha$  response against subgenomic replicons of HCV in Huh7 and HeLa cells is investigated. The following conclusions can be  
25        drawn from these investigations. First, it can be concluded that IFN- $\alpha$  can inhibit HCV replication by both noncytopathic and cytopathic mechanisms. These results demonstrating that SL1 cells treated with IFN- $\alpha$  (100 IU/ml) underwent programmed cell death raised the  
30        question of whether apoptosis contributes to the rapid decline of HCV RNA levels observed during the first 48 h of IFN therapy (Neumann, A. U., et al., 1998. Science 282:103-107). The answer depends on whether HeLa or Huh7 cells mimic the scenario in HCV-infected hepatocytes in  
35        vivo. It is known from this and other studies that Huh7

cells exhibit an attenuated response to dsRNA and cannot induce an apoptotic program (results not shown) (Keskinen, P., et al., 1999. *Virology* 263:364-375; Lanford, R. E., et al., 2003. *J. Virol.* 77:1092-1104; McNair, A. N., et al., 1994. *J. Gen. Virol.* 75:1371-1378). In contrast, HeLa cells respond to dsRNA in a fashion similar to that in which primary chimpanzee hepatocytes respond. For example, treatment of chimpanzee primary hepatocyte cultures with poly(I:C) led to the induction of IFN- $\beta$ , as shown in this report with HeLa cells (Figure 12) (Lanford, R. E., et al., 2003. *J. Virol.* 77:1092-1104). Therefore, it is likely that HeLa cells represent a more physiological model for hepatocytes in terms of IFN response than Huh7 cells. It was notable that only a fraction of SL1 cells died after treatment with IFN- $\alpha$ . One possibility is that apoptosis was induced in cells that replicated above-average levels of HCV RNA. In support of this possibility, reduction of viral levels by treatment with heat or HCV RNA polymerase inhibitors reduced the number of apoptotic cells after IFN- $\alpha$  treatment (Figure 8 and results not shown). Based on these results it was concluded that HeLa cells did not undergo apoptosis by default after IFN- $\alpha$  treatment. In fact, it appears that apoptosis is a hallmark of HCV-replicating HeLa cells, because HeLa cells replicating Kunjin virus RNA remained viable after IFN treatment. Finally, it appears that, the observation reported here represents the first example of IFN- $\alpha$ -induced apoptosis of cells replicating an apparently noncytolytic RNA virus.

Second, it can be concluded that the noncytopathic response can occur independently of dsRNA-dependent pathways. Although these results showed that dsRNA response pathways were at least partially functional in normal and HCV-replicating Huh7 cells and were intact in

HeLa cells, as indicated by poly(I:C)-induced phosphorylation of eIF-2 $\alpha$  and IFN- $\beta$  gene transcription, viral replication per se did not induce such responses (Figure 12). The expression of the vaccinia virus E3L protein or treatment of cells with the kinase inhibitor 2-AP had no measurable effect on the IFN response against the HCV replicon (Figure 11 and Table 5). These observations were consistent with the notion that dsRNA-dependent antiviral pathways, such as PKR and RNase L pathways, were not involved in IFN-induced inhibition of HCV replication in Huh7 cells. Whether they play a role in HeLa cells is not yet known. Efforts to express E3L in SL1 cells were not successful due to the apparent toxicity of the protein, and treatment of HeLa cells with 2-AP for more than 12 h induced apoptosis (results not shown). Importantly, it is not yet known whether IFN-induced apoptosis in HCV-expressing cells is dependent on PKR or other dsRNA-dependent pathways (see below).

In summary, the results showed that, while both Huh7 and HeLa cells were competent to activate dsRNA-dependent signal transduction pathways, HCV replication alone was not sufficient to induce a detectable dsRNA response in these cells. This result could indicate that dsRNA either did not accumulate during HCV replication or was not accessible to PKR and other dsRNA binding proteins.

Third, it can be concluded that HCV replication can induce innate immune pathways. In SL1 and other HCV-expressing HeLa cell lines (results not shown), but not in normal HeLa cells, IFN- $\alpha$  induced the expression of IFN- $\beta$ . This indicates that HCV replication activated an unknown cellular factor, perhaps a viral activated kinase as proposed by Smith and colleagues (Smith, E. J., et al., 2001. J. Biol. Chem. 276:8951-8957), that, in turn, activated one or more transcription factors required for IFN- $\beta$  transcriptional activation. Candidates include



IRF3, NF- $\kappa$ B, and ATF-2 (Figure 10). Because normal HeLa cells did not undergo apoptosis after IFN- $\alpha$  treatment, it can be concluded that HCV expression directs the IFN- $\alpha$  response into a cytopathic process.

5 Fourth, it can be concluded that the antiviral activity of IFN- $\alpha$  against HCV depends, in part, on functional proteasomes. How IFN-induced antiviral programs inhibit viral replication noncytopathically is not yet understood. The results shown here demonstrate  
10 that, for HCV replication, proteasomes were required for this process. However, the idea that proteasomes were directly involved in inhibition of HCV replication by increasing the turnover of replication complexes or viral proteins was not supported by these results. Instead  
15 evidence was obtained that induction of the IFN response was dependent on degradation of one or several proteins. Previously, it has been shown (Li, X. L., and B. A. Hassel. 2001. Cytokine 14:247-252) that proteasome inhibitors attenuated the induction of certain IFN-  
20 stimulated genes. Because epoxomicin and lactacystin did not inhibit induction of Mx-A (results not shown), which is dependent on activation of the Jak-STAT pathway for the formation of ISGF3, proteasomes might be involved in the induction of the second-wave IFN-stimulated genes.  
25 Such a model is consistent with results published previously by Li and Hassel (Li, X. L., and B. A. Hassel. 2001. Cytokine 14:247-252), who found that treatment of cells with proteasome inhibitors did not inhibit phosphorylation of STAT1 and binding of ISGF3 to DNA. As  
30 a consequence of these results, the number of IFN-induced genes that play a role in inhibition of HCV replication by IFN- $\alpha$  can be reduced to those that are repressed by epoxomicin.

An important implication of these results for  
35 clinical IFN- $\alpha$  therapy and the pathogenesis of HCV

infections is that besides the noncytopathic antiviral effects, IFN- $\alpha$  might also induce apoptosis of HCV-infected hepatocytes. At first glance, this possibility might be discounted because drug-induced cell death could lead immediately to the destruction of the infected liver. However, it is possible that only a fraction of hepatocytes express levels of HCV high enough to activate an apoptotic program in the presence of the high levels of IFN- $\alpha$  that are used for antiviral therapy. In this scenario cell death would occur unnoticed. An important consequence of such a scenario would be that cell killing could play a major role in the recovery from chronic HCV infections, similar to the situation in natural recovery from transient infections with woodchuck hepatitis virus, a model for hepatitis B virus infections (Guo, J. T., et al., 2000. J. Virol. 74:1495-1505).

**Table 6. Listing of Sequence ID Numbers**

<b>Sequence</b>	<b>Sequence ID Number</b>
I <sub>377</sub> /NS3-3'	SEQ ID NO:1
pZS1	SEQ ID NO:2
pZS2	SEQ ID NO:3
pZS4	SEQ ID NO:4
pZS5	SEQ ID NO:5
pZS6	SEQ ID NO:6
pZS8	SEQ ID NO:7
pZS10	SEQ ID NO:8
pZS11	SEQ ID NO:9
pZS12	SEQ ID NO:10
pZS15	SEQ ID NO:11
pZS20	SEQ ID NO:12
pZS25	SEQ ID NO:13
pZS45	SEQ ID NO:14
Mx-A cDNA primer #1	SEQ ID NO:15
Mx-A cDNA primer #2	SEQ ID NO:16

While certain preferred embodiments of the present  
5 invention have been described and specifically  
exemplified above, it is not intended that the invention  
be limited to such embodiments. Various modifications  
may be made to the invention without departing from the  
scope and spirit thereof as set forth in the following  
10 claims.